UNIVERSITY OF ALBERTA

INVESTIGATIONS INTO THE METABOLISM OF TWO NOVEL NEUROPROTECTIVE AGENTS BY HUMAN CYTOCHROME P450 ENZYMES

BY

KATHERINE A. RITTENBACH ©

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
IN
DEPARTMENT OF PSYCHIATRY

EDMONTON, ALBERTA
FALL 2007

Library and
Archives Canada

Published Heritage
Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et
Archives Canada

Direction du
Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-33052-4
Our file Notre référence
ISBN: 978-0-494-33052-4

NOTICE:
The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:
L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

This thesis is dedicated to my parents, Shirley Ann and Donald Dale Rittenbach.

I am grateful for their influence and love, which helped shape who I am today.

I will always miss them.

Abstract

(-)-Deprenyl, an antiParkinson drug, demonstrates neuroprotective efficacy in several models of neurotoxicity, but has the disadvantage of being metabolized to the potential neurotoxins(-)methamphetamine and (-)-amphetamine. Two novel analogues of (-)-deprenyl which would not be metabolized to amphetamines, N-methyl,N-propargyl-β-phenylethylamine (MPPE) and N-methyl,N-propargylphentermine (MPPT), have been synthesized in our laboratories and have demonstrated neuroprotection. Rapid and sensitive analytical methods for the proposed metabolites of MPPE and MPPT were developed and applied to metabolism studies using human liver microsomes (HLMs) and cDNA-expressed individual human cytochrome P450 (CYP) enzymes. HLM panel studies demonstrated that both drugs are metabolized in similar patterns to (-)-deprenyl, and the rates of formation of the primary metabolites, N-methylphenylethylamine, N-propargylphenylethylamine, N-methylphentermine and N-propargylphentermine were determined. HLM panels were also used to demonstrate metabolism of the primary metabolites to secondary metabolites. Both N-methylphentermine and N-propargylphentermine were metabolized to phentermine, but only N-propargylphenylethylamine was metabolized to phenylethylamine (PEA).

A fluorogenic screen established interactions of MPPE and MPPT and their metabolites with individual CYP enzymes. The CYP enzymes inhibited strongly by MPPE or MPPT were investigated further to determine if they catalyzed the metabolism of the drugs to the primary metabolites. The effects of

CYPs 2B6, 2C19 and 2D6 on metabolism of MPPE, MPPT and (-)-deprenyl were quite different. CYP2B6 catalyzed N-demethylation and N-depropargylation of all three compounds, though at significantly different rates; CYP2C19 catalyzed N-demethylation of all three compounds but N-depropargylation of only MPPT; and CYP2D6 catalyzed N-depropargylation of both (-)-deprenyl and MPPE but only catalyzed N-demethylation of (-)-deprenyl.

These studies resulted in the development of sensitive, rapid assays for metabolites and characterized the metabolism of the novel neuroprotective agents MPPE and MPPT. The data obtained demonstrate that CYP-mediated metabolism of N-propargylphenylethylamines can be affected markedly by simple chemical modifications such as removal or addition of a methyl group at the side chain α carbon. Such information will be very useful in future studies *in vivo* on the metabolism and drug-drug interactions involving MPPE and MPPT.

ACKNOWLEDGEMENTS

I wish to acknowledge the help and guidance of my supervisor, Dr G.B. Baker throughout this program. His knowledge, integrity and dedication are inspiring and I will forever be grateful for his understanding and kindness during my dad's illness.

I would like to thank Drs. Kathryn Todd, Kathleen Hegadoren, Ronald Coutts and Adil Nazarali for giving their time.

There have been many people in my life who have helped me on my path and while I cannot name them all I do thank them all. My family has been a source of encouragement and support my entire life. Ann and Dale have laughed and fought with me the whole way through. Donna, Trina, Mark, Kim, Steve, Jason, Micah, Tony, Brock and Beka have taught me so many of life's valuable lessons in such a short time and I look forward to learning more from them.

For supporting me both in and out of the lab, I would like to thank Lauren, Melissa, Vic, Joelle, and Jacynthe. All of the members of the NRU have helped my studies in countless ways and I am truly grateful to all of them.

For pushing me past my comfort zone and challenging me to continue to grow, I thank Kathy.

CHAPTER 1 INTRODUCTION

1.1. Introduction 2
1.2. Parkinson's Disease 5
1.3. Other Uses for Deprenyl 7
1.4. Neuroprotection 10
  1.4.1. Neuroprotection in Neurotoxicity Models 10
  1.4.2. Mechanism of Action 12
1.5. (-)-Deprenyl Metabolism 16
1.6. Rasagiline 19
1.7. MPPE and MPPT 21
1.8. Xenobiotic Metabolism 25
  1.8.1. Cytochromes P450 26
  1.8.2. CYP structure and mechanism of action 29
  1.8.3. Factors affecting xenobiotic metabolism 30
  1.8.4. Individual enzymes of importance in deprenyl metabolism 34
    1.8.4.1. CYP2B6 34
    1.8.4.2. CYP2C19 35
    1.8.4.3. CYP2D6 36
  1.8.5. Drug Interactions 36
    1.8.5.1. Methods for studying drug interactions 37
  1.8.6 Enzyme Kinetics 39
1.9 Analytical techniques relevant to this thesis 41
  1.9.1 Gas Chromatography 41

1.9.1.1 Instrumentation 42
1.9.1.2 Derivatization 45
1.9.2 High performance liquid chromatography 47
1.9.2.1 Instrumentation 48
1.9.3 Mass spectrometry 49
1.9.3.1 Instrumentation 50
1.10 Objectives and hypothesis 50
1.11 References 52

CHAPTER 2 A RAPID, SELECTIVE ELECTRON-CAPTURE GAS CHROMATOGRAPHIC PROCEDURE FOR ANALYSIS OF METABOLITES OF N-METHYL,N-PROPARGYLPHENYLETHYLAMINE, A POTENTIAL NEUROPROTECTIVE AGENT.

2.1 Introduction 65
2.2 Methods 67
2.2.1 Materials 67
2.2.2 Human liver microsome incubations 70
2.2.3 Sample preparation, derivatization and analysis 71
2.2.4 GC-ECD analysis 72
2.2.5 GC-MS analysis 72
2.3 Results 72
2.4 Discussion 80
2.5 References 83

CHAPTER 3 METABOLISM OF N-METHYL,N-PROPARGYLPHENYLETHYLAMINE: STUDIES WITH HUMAN LIVER MICROSOMES AND cDNA-EXPRESSED CYTOCHROME P450 (CYP) ENZYMES.

3.1 Introduction 87

3.2 Methods 90

3.2.1 Materials 90

3.2.2 Incubations with human liver microsomes 91

3.2.3 Sample preparation, derivatization and analysis 92

3.2.4 Fluorogenic screen for interactions of MPPE with CYP enzymes 93

3.2.5 Studies on metabolism of MPPE by cDNA-expressed CYP enzymes 95

3.3 Results 96

3.4 Discussion 102

3.5 References 107

CHAPTER 4 INVOLVEMENT OF CYTOCHROMES P450 2D6, 2B6 AND 2C19 IN THE METABOLISM OF (-)-DEPRENYL AND N-METHYL,N-PROPARGYLPHENYLETHYLAMINE.

4.1 Introduction 112

4.2 Methods 117

4.2.1 Materials 117

4.2.2 Studies on metabolism of MPPE using cDNA-expressed CYP enzymes 117

4.2.3 Sample preparation, derivatization and analysis 119

4.2.4 Statistics 119

4.3 Results 119

4.4 Discussion 121

4.5 References 125

CHAPTER 5 A RAPID, SENSITIVE ELECTRON-CAPTURE GAS CHROMATOGRAPH PROCEDURE FOR ANALYSIS OF METABOLITES OF N-METHYL,N-PROPARGYLPHENTERMINE, A POTENTIAL NEUROPROTECTIVE AGENT.

5.1 Introduction 130

5.2 Methods 132

  5.2.1 Materials 132

  5.2.2 Human liver microsome incubations 135

  5.2.3 Sample preparation, derivatization and analysis 136

  5.2.4 GC-ECD analysis 137

  5.2.5 GC-MS analysis 138

5.3 Results 138

5.4 Discussion 145

5.5 References 150

CHAPTER 6 METABOLISM OF N-METHYL,N-PROPARGYLPHENTERMINE: STUDIES WITH HUMAN LIVER MICROSOMES AND cDNA EXPRESSED CYTOCHROME P450 (CYP) ENZYMES.

6.1 Introduction 155

6.2 Materials and methods 158

6.2.1 Materials 158

6.2.2 Fluorogenic screen for interaction of MPPT with CYP enzymes 159

6.2.3 Human liver microsome incubations 161

6.2.4 Sample preparation, derivatization and analysis 162

6.2.5 Studies on metabolism of MPPT by cDNA-expressed CYP enzymes 163

6.3 Results 163

6.4 Discussion 167

6.5 References 170

CHAPTER 7 INVOLVEMENT OF CYTROCHROMEs P450 2D6, 2B6 AND 2C19 IN THE METABOLISM OF (-)-DEPRENYL AND N-METHYL,N-PROPARGYLPHENTERMINE

7.1 Introduction 174

7.2 Methods 179

7.2.1 Materials 179

7.2.2 Studies on metabolism of MPPT using cDNA-expressed CYP enzymes 179

7.2.3 Sample preparation, derivatization and analysis 180

7.2.4 Statistics 181

7.3 Results 181

7.4 Discussion 185

7.5 References 186

CHAPTER 8 GENERAL DISCUSSION

8.1 Discussion 190

8.2 Future directions 205

8.3 References 208

TABLE INDEX

Chapter 1

Table 1.1 Effective symptomatic treatement for early Parkinson's Disease. 6

Table 1.2 The 57 human CYP enzymes and their primary substrates. 27

Chapter 2

Table 2.1 Mean recovery and reliability of assay. 78

Table 2.2 Concentration of metabolites of MPPE formed during incubations with human liver microsomes. 81

Chapter 3

Table 3.1 Fluorogenic CYP substrates and excitation and emission wavelengths. 94

Chapter 5

Table 5.1 Recovery data. 143

Table 5.2 Intra-assay reliability C.V. data. 144

Table 5.3 Inter-assay reliability C.V. data. 146

Table 5.4 Standard curve data. 147

Chapter 6

Table 6.1 Fluorogenic CYP substrates and excitation and emission wavelengths. 160

Chapter 8

Table 8.1 Qualitative summary of interactions in fluorogenic screen. 201

Table 8.2 Summary of CYP-mediated metabolism of MPPE, MPPT and (-)-deprenyl by CYPs 2B6, 2C19 and 2D6 202

FIGURE INDEX

Chapter 1

Figure 1.1 Structure of N-methyl,N-propargylamphetamine (-deprenyl) 3

Figure 1.2 Major metabolic routes of (-)-deprenyl. 17

Figure 1.3 Identified hydroxylated and N-oxide metabolites of (-)-deprenyl 18

Figure 1.4 Structure of rasagiline. 20

Figure 1.5 Structures of N-methyl,N-propargyl-2-phenylethylamine (MPPT) and N-methyl,N-propargylphentermine (MPPT) 22

Figure 1.6 Metabolism of MPPE. 23

Figure 1.7 Metabolism of MPPT. 24

Figure 1.8 Simplified CYP P450 catalytic cycle. 31

Figure 1.9 Competitive, noncompetitive and uncompetitive inhibition schematics 33

Equation 1.1 Michaelis-Menton equation. 40

Figure 1.10 Schematic of a gas chromatograph. 43

Figure 1.11 Schematic of derivatization of secondary amines with PFBC and PFBSC 46

Chapter 2

Figure 2.1 Metabolism of deprenyl. 66

Figure 2.2 Proposed metabolism of MPPE. 68

Figure 2.3 Representation of derivatization of phenylethylamines with PFBSC 69

Figure 2.4 Typical gas chromatagraph traces of derivatized MPPE metabolites 74

Figure 2.5 Mass spectral fragmentation of β-phenylethylamine derivatized with PFBSC 75

Figure 2.6 Mass spectral fragmentation of N-methylphenylethylamine derivatized with PFBSC 76

Figure 2.7 Mass spectral fragmentation of N-propargylphenylethylamine with PFBSC 77

Figure 2.8 Typical standard curves for PEA, N-methylPEA and N-propargylPEA 79

Chapter 3

Figure 3.1 Proposed CYP-mediated metabolism of MPPE. 89

Figure 3.2 Inhibition by MPPE of metabolism of fluorogenic substrates 97

Figure 3.3 Rates of metabolism of MPPE by CYP 2B6, 2C19 and 2D6 98

Figure 3.4 Time courses for CYP-mediated formation of N-methylPEA and N-propargylPEA 100

Figure 3.5 Kinetic plots for CYP-mediated generation of N-methylPEA and N-propargylPEA 101

Figure 3.6 Metabolism of MPPE primary metabolites. 103

Chapter 4

Figure 4.1 Metabolism of (-)-deprenyl. 113

Figure 4.2 Metabolism of MPPE. 116

Figure 4.3 Results of incubation of MPPE and (-)-deprenyl (500 μM each) with cDNA-expressed CYP enzymes 120

Figure 4.4 Results of incubation of MPPE and (-)-deprenyl (50 μM each) with cDNA-expressed CYP enzymes 122

Chapter 5

Figure 5.1 Metabolism of (-)-deprenyl. 131

Figure 5.2 Proposed metabolism of MPPT. 133

Figure 5.3 Representation of derivatization of phentermines with PFBC. 135

Figure 5.4 Representative GC trace of derivatization of compounds of interest. 139

Figure 5.5 Mass spectral fragmentation of phentermine derivatized with PFBC. 140

Figure 5.6 Mass spectral fragmentation of N-methylphentermine derivatized with PFBC. 141

Figure 5.7 Mass spectral fragmentation of N-propargylphentermine derivatized with PFBC. 142

Figure 5.8 Representative GC trace of derivatized metabolites of 500 μM MPPT incubated with HLMs. 149

Chapter 6

Figure 6.1 Metabolism of N-methyl,N-propargylphentermine (MPPT). 157

Figure 6.2 Inhibition by MPPT of metabolism of fluorogenic substrates. 164

Figure 6.3 Kinetic plots for CYP-mediated formation of N-methylPHEN and N-propargylPHEN. 166

Figure 6.4 Secondary metabolism of MPPT. 168

Chapter 7

Figure 7.1 Metabolism of (-)-deprenyl. 175

Figure 7.2 Metabolism of MPPT. 178

Figure 7.3 CYP2B6 mediated metabolism of MPPT and (-)-deprenyl. 182

Figure 7.4 CYP2C19 mediated metabolism of MPPT and (-)-deprenyl. 183

Figure 7.5 CYP2D6 mediated metabolism of MPPT and (-)-deprenyl. 184

Abbreviation List

6-OHDA	6-hydroxydopamine
μl	microliter
μm	micrometer
μM	micromolar
AADC	aromatic acid decarboxylase
AB	Alberta
AD	Alzheimer's Disease
ADHD	attention deficit hyperactivity disorder
ADI	adverse drug interactions
ANOVA	analysis of variance
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CV	coefficients of variance
CYP	cytochrome P450
Deprenyl	N-methyl,N-propargylamphetamine
DNA	deoxyribonucleic acid
DSP-4	N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine
ECD	electron capture detector
G6P	glucose-6-phosphate
G6PD	glucose-6-phosphate dehydrogenase
GC	gas chromatograph
GC-ECD	gas chromatography with electron capture detection
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HCl	hydrochloric acid
HLMs	human liver microsomes
HPLC	high performance liquid chromatography
IS	internal standard
K_I	inhibitor dissociation constant
K_M	concentration of substrate that results in an initial velocity of half of V_{max}
L-DOPA	levo-3,4-dihyroxyphenylalanine
LOD	limit of detection
M	molar
m	meter
MA	Massachusetts
MAO	monoamine oxidase
mg	milligram
ml	milliliter
mM	millimolar
mm	millimeter
min	minute
MO	Missouri
MPP^+	1-methyl-4-phenylpyridinium
MPPE	N-methyl,N-propargyl-2-phenylethylamine

MPPT	N-methyl,N-propargylphentermine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger ribonucleic acid
MS	mass spectrometer/mass spectroscopy
m/z	weight to charge ratio
Na^+,K^+-ATPase	sodium, potassium adenosine triphosphatase
NA	noradrenaline
NADP	nicotinamide adnenine dinucleotide phosphate
NADPH	nicotinamide adnenine dinucleotide phosphate, reduced
ng	nanogram
NGF	nerve growth factor
Ni	nickel
nM	nanomolar
nm	nanometer
NMDA	N-methyl-D-aspartate
N-methylPEA	N-methylphenylethylamine
N-methylPHEN	N-methylphentermine
N-propargylPEA	N-propargylphenylethylamine
N-propargylPHEN	N-propargylphentermine
ON	Ontario
PD	Parkinson's Disease
PEA	β-phenylethylamine
PFBC	pentafluorobenzyol chloride
PFBSC	pentafluorobenzenesulfonyl chloride
PHEN	phentermine
PM	poor metabolizer
pMol	picomole
USA	United States of America
rasagiline	N-propargyl-1R-aminaindan
rpm	revolutions per minute
RT	retention time
[S]	substrate concentration
SCOT	support coated open tubular
SD	standard deviation
SEM	standard error of the mean
SOD	superoxide dismutase
TD	thiamine deficiency
UDP	uridine 5'-diphosphate
URM	ultrarapid metabolizer
UV/VIS	ultraviolet/visible
v	velocity of formation
V_{max}	maximum rate of metabolite formation
v/v	volume per volume
WCOT	wall coated open tubular

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Neuroprotection is defined as the "mechanisms and strategies used to protect against neuronal injury or degeneration in the central nervous system (CNS) following acute disorders or as a result of chronic neurodegenerative diseases" (Hill, 2006). Neurorescue differs from neuroprotection in that neurorescue focuses on treating damaged neurons that would normally die, and reversing the process after the insult, whereas neuroprotection occurs before or during the insult with the goal of preventing damage. The goal of both is to minimize neuronal death after an insult to the CNS, minimizing dysfunction in patients after insults, whether the insult is a stroke or neurodegenerative diseases such as Parkinson's Disease or Alzheimer's Disease. While there are many neuroprotective agents available or under investigation (Hill, 2006), the mechanisms of action for all of them are not fully understood. The lack of knowledge of mechanisms of action makes metabolism studies important and relevant due to the possibility that metabolites of the agent of interest may be contributing to the response. The project described in this thesis will investigate the metabolic pathways of two novel neuroprotective compounds that are structural analogues of (-)-deprenyl and compare their metabolism with that of (-)-deprenyl.

Deprenyl (N-methyl,N-propargylamphetamine) (Figure 1.1) is a chiral amine that was first synthesized in 1962 by Esceri for Chinoin Pharmaceutical Works, Hungary. In 1967 it was determined that the (-) isomer was more effective than the (+) isomer at inhibiting monoamine oxidase (MAO)

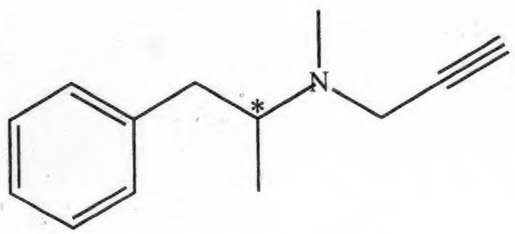


Figure 1.1. Structure of N-methyl,N-propargylamphetamine (deprenyl). The chiral centre is indicated by an asterisk.

(Magyar, K. *et al.*, 1967), and by 1972 it was known that (-)-deprenyl (selegiline) selectively and irreversibly inhibited MAO-B (Knoll and Magyar, 1972). (-)-Deprenyl is readily absorbed and rapidly enters the brain and spinal cord after oral administration (Gerlach *et al.*, 1996). It was originally investigated as an antidepressant because it was thought that it might have the antidepressant effects of other MAO-inhibitors such as iproniazid, tranylcypramine and phenelzine while not having the "cheese effect" that had reduced the use of these drugs (Youdim *et al.*, 2006). The "cheese effect" results from excessive stimulation of the perifpheral nervous system by dietary tyramine (found in aged cheese and wine). MAO-A in the gastro-intestinal tract metabolizes dietary tyramine preventing excessive stimulation, however oral doses of MAO-A inhibitors inhibit the gastro-intestinal tract MAO-A resulting in increased systemic concentrations of tyramine. (-)-Deprenyl was not particularly effective as an antidepressant at doses which primarily inhibited MAO-B, though recent studies show that a transdermal patch containing (-)-deprenyl is effective as an antidepressant (Patkar *et al.*, 2006). Transdermal administration increases the concentration of (-)-deprenyl versus the primary metabolites compared to oral adminstration. (-)-Deprenyl began to be used in Parkinsons Disease (PD) after reports that it was effective in delaying death of patients with this disease when coadministered with levo-3,4-dihydroxyphenylalanine (L-DOPA) were first published in 1985 by Birkmayer and his colleagues (Birkmayer *et al.*, 1985).

1.2 PARKINSON'S DISEASE

Parkinson's disease is a progressive neurological disorder that was first described in detail by James Parkinson in 1817, although it was probably reported thousands of years prior (Paolini *et al.*, 2004). The cause of the disease is still unknown and there is no curative treatment (Guttman *et al.*, 2003).

Diagnosis of PD is based on clinical presentation because definitive diagnosis can only be made upon autopsy. The obvious clinical signs are unilateral tremor at rest (Guttman, *et al.*, 2003) and bradykinesia, the inability to initiate movement. The principal pathological findings upon autopsy are: loss of dopaminergic neurons in the substantia nigra pars compacta; Lewy bodies (intracellular proteinaceous inclusions); and marked reductions in striatal dopamine (Schapira and Olanow, 2004).

While PD can not currently be cured, there are symptomatic treatments. Patients with early stages of Parkinson's will respond positively to many drugs (Table 1.1), but as the disease progresses, L-DOPA is the "gold standard". L-DOPA is a precursor of dopamine that crosses the blood-brain barrier and is converted into dopamine by aromatic amino acid decarboxylase (AADC) in the nigrostriatal neurons, compensating for the decreases in endogenous dopamine (Olanow, 2004). However, L-DOPA does not slow the progression of the deterioration and, further, after more than five years of treatm[illegible] [illegible] therapy causes motor symptoms in as many as 80% of patients (Shults, 2003; Olanow, 2004). Many patients also develop symptoms over time that do not respond to L-DOPA therapy; these symptoms include freezing periods, falls, depression and

Drug or drug class	**Mechanism of action**
Anticholinergics	Block acetylcholine receptors
Amantadine	Blocks NMDA receptors and acetylcholine receptors and promotes release of dopamine
Dopamine agonists	Directly stimulate dopamine receptors
Monoamine oxidase inhibitors	Block MAO-B metabolism of dopamine

Table 1.1. Effective symptomatic treatment for early Parkinson's Disease (NMDA = N-methyl-D-aspartate; MAO-B = monoamine oxidase B) [modified from (Guttman, *et al.*, 2003)].

dementia (Olanow, 2004). Selegiline [(-)-deprenyl] was one of the first therapies observed to delay the progress of PD (Riederer and Lachenmayer, 2003) though there has been extensive debate about whether this is symptomatic relief or neuroprotection (Shults, 2003).

1.3 OTHER USES FOR DEPRENYL

Researched uses for (-)-deprenyl are diverse and include studies in Alzheimer's disease (AD). AD is characterized by gradual and continuous decline in cognitive function, behavior and independence due to dementia, the cardinal symptom (Grossberg, 2003). A decrease in acetylcholine levels in the brain of Alzheimer's patients is commonly observed and most of the treatments approved for use in this disorder are cholinesterase inhibitors (Grossberg, 2003). Noncholinergic treatments that have been studied include Vitamin E, estrogen, lipid-lowering agents, the N-methyl-D-aspartate (NMDA) glutamate receptor antagonist memantine and (-)-deprenyl (Sano, 2003). (-)-Deprenyl has shown beneficial effects in Alzheimer patients. One study showed (-)-deprenyl delayed the primary endpoint by over 200 days (in this study the primary endpoint was time to reach a clinically meaningful endpoint--death, institutionalization or loss of 2 of 3 basic activities of daily living) (Sano *et al.*, 1997). A second study demonstrated improved mini mental status examination scores for patients on (-)-deprenyl in a long term (24 weeks), double-blind, placebo controlled study (Filip *et al.*,1999). A post-mortem study of AD patient brain tissues showed similar lesions in those treated with (-)-deprenyl compared to those not treated with (-)-deprenyl, however the treated patients had significantly higher mini-mental state examinations (Alafuzoff *et al.*, 2000). Previous studies focused on MAO-B inhibition as the mechanism of action, but recent *in vivo* studies suggested a novel mechanism of action. Treatment with (-)-deprenyl inhibits the formation of ß-amyloid fibrils *in vitro* and also destabilizes preformed fibrils (Ono *et al.*, 2006) which could help prevent AD as fibril formation in the brain is a cardinal finding in AD patients.

(-)-Deprenyl increases dopamine concentrations in the brain by inhibiting MAO-B (Thomas, 2000), which has led to the hypothesis that this drug could be an effective treatment for both cocaine and methamphetamine addiction and abuse by stimulating the reward mechanisms in the brain proposed to be involved in the "high" experienced from these drugs (Bartzokis *et al.*, 1999; Newton *et al.*, 1999; Kosten *et al.*, 2002). The studies to date are not conclusive. Studies have shown that administration of (-)-deprenyl attenuated some subjective effects produced by cocaine, such as the "high" and "stimulation" (Houtsmuller *et al.*, 2004). It may attenuate the "high" due to cocaine and methamphetamine by elevating the baseline concentration of dopamine, which is decreased in cocaine and methamphetmine addicts, and hence the difference between dopamine concentrations with and without illicit drug administration would be smaller, resulting in the smaller subjective "high". Other studies have shown that pretreatment with (-)-deprenyl failed to alter drug-seeking behavior maintained by (+)-amphetamine (Yasar *et al.*, 2006).

(-)-Deprenyl is also a promising compound for the treatment of attention deficit hyperactivity disorder (ADHD) in children (Akhondzadeh *et al.*, 2003). Currently the stimulant methylphenidate is the most effective therapy for this disorder, but others such as (+)-amphetamine are also used (Boix *et al.*, 1998). (-)-Deprenyl may be effective in this disorder because altered reinforcement processes due to low dopamine levels in the brain is a proposed cause of the behavioral disturbances seen in ADHD and (-)-deprenyl increases dopamine levels (Boix, *et al.*, 1998). An alternate theory is that decreased dopamine and noradrenaline reduce the inhibitory influences of frontal cortical activity on subortical structures (Akhondzadeh, *et al.*, 2003). In a double-blind, randomized and controlled study of children, (-)-deprenyl was effective and it was suggested that it may have advantages in children unable to tolerate the side-effects of stimulant treatments (Akhondzadeh, *et al.*, 2003).

Another disorder for which (-)-deprenyl can potentially be useful is schizophrenia, specifically the negative symptoms of the disease. Negative symptoms include affective flattening, alogia, anhedonia and avolition (Fohey *et al.*, 2007). These negative symptoms are less responsive than positive symptoms (delusions and hallucinations) to many antipsychotics and are important in the global dysfunction of the disease (Fohey, *et al.*, 2007). Dopaminergic hypofunction has been proposed as an underlying cause of negative symptoms of schizophrenia, leading to the study of MAO inhibitors for the attenuation of these symptoms (Bodkin *et al.*, 2005). Typically the dietary restrictions and overstimulation resulting from complete MAO inhibition have limited the usefulness of these drugs in schizophrenia. However, in a placebo-controlled double blind study, patients taking (-)-deprenyl at doses that are MAO-B selective along with their normal antipsychotic showed improvements in negative symptoms (Bodkin, *et al.*, 2005). Although studies up to this point have small sample sizes, the results of a review of all of them show that (-)-deprenyl is a promising adjunctive therapy for the negative symptoms of schizophrenia (Murphy *et al.*, 2006).

(-)-Deprenyl has been investigated as a treatment for a wide variety of disorders, and shows promise in many of them. The diversity of disorders reflects the multiple mechanisms of action hypothesized for (-)-deprenyl.

1.4 NEUROPROTECTION

Various mechanisms of action have been proposed for the neuroprotective effects of (-)-deprenyl observed in a variety of neurotoxicity models.

1.4.1 Neuroprotection in neurotoxicity models

The thiamine deficiency (TD) model of Wernicke's encephalopathy produces reproducible, selective neuronal loss in the thalamus, inferior colliculus and inferior olive, which is significantly decreased by treatment by (-)-deprenyl (Todd and Butterworth, 1998). Cell death in TD is suggested to be due to cellular damage produced by reactive oxygen species (Todd and Butterworth, 1998). (-)-Deprenyl administration may be neuroprotective in this model by decreasing the production of these free radicals (Sowa *et al.*, 2004).

DSP-4 (N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine) is a neurotoxin that induces lesions in brain regions innervated with noradrenaline (NA) axons of locus coeruleus origin (hippocampus and cortex) and produces eventual loss of NA cell bodies in the locus coeruleus (Fritschy and Grzanna, 1991). (-)-Deprenyl prevents DSP-4 degeneration of both the axons and cell bodies (Yu *et al.*, 1994; Zhang *et al.*, 1995). Early work suggested that protection was due to MAO-B inhibition (Gibson, 1987), although other work contradicted this theory (Finnegan *et al.*, 1990). The mechanism of neuroprotection by (-)-deprenyl in DSP-4 models of neurotoxicity is still unknown.

The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces neuropathic abnormalities in humans and nonhuman primates that are similar to those observed in idiopathic PD. These include the loss of dopaminergic neurons and resulting tremors. MPTP itself is not neurotoxic, but MAO-B catalyzes metabolism of this compound to 1-methyl-4-phenylpyridinium (MPP^+), which is neurotoxic (Markey *et al.*, 1984). (-)-Deprenyl administration blocks the development of this disorder through MAO-B inhibition. However, several investigators have shown neuroprotection by (-)-deprenyl in the MPTP model of neurotoxicity at (-)-deprenyl concentrations that do not block MAO-B catalyzed metabolism, and in cells that do not contain MAO-B, which suggests a second unknown pathway (Ebadi *et al.*, 2002).

A model of excessive glutamatergic activity is created by the administration of N-methyl-D-aspartate (NMDA), a glutamate receptor agonist. This model is useful because excessive glutamate is considered a key stage of neurodegeneration in glaucoma and a cause of cell death after hypoxic/ischemic insults (Takahata *et al.*, 2003). (-)-Deprenyl administration prior to insult with NMDA has been shown to protect retinal neurons (Takahata, *et al.*, 2003). These results may be due to anti-apoptotic actions of (-)-deprenyl, as administration was able to protect the layer of cell bodies, but not the layer of dendrites (Takahata, *et al.*, 2003). However MAO-B inhibition has not been ruled out as the protective mechanism in this model.

1.4.2 Mechanisms of action

The mechanism of action of (-)-deprenyl in PD is debated. Originally it was used in PD in an attempt to increase the concentration of dopamine by inhibiting dopamine metabolism by MAO-B. Rapid symptomatic relief from PD due to MAO-B inhibition is well accepted (Berry *et al.*, 1994), although this relief has been proposed to result from the inhibition of β-phenylethylamine (PEA) metabolism rather than dopamine metabolism (Berry *et al.*, 1994). Chronic effects of (-)-deprenyl administration include the complete inhibition of MAO-B, significant inhibition of MAO-A and altered messenger ribonucleic acid (mRNA) expression for a variety of proteins including an increase in glial aromatic acid decarboxylase (AADC) (Berry, *et al.*, 1994). This increase in AADC mRNA would presumably result in increased levels and activity of AADC and ultimately an increase in formation of dopamine from L-DOPA by AADC in Parkinson's patients administered both L-DOPA and (-)-deprenyl (Berry, *et al.*, 1994).

(-)-Deprenyl appears to act as an anti-oxidant through at least two mechanisms of action. MAO-B inhibition decreases the formation of damaging oxygen radical species by blocking normal metabolism of dopamine which can lead to the formation of oxygen radical species (Magyar and Szende, 2004). (-)-Deprenyl provides further protection from oxidative stress through the upregulation of anti-oxidant proteins (Nakaso *et al.*, 2006). Studies show that long term treatment with (-)-deprenyl increases the production and/or activity of superoxide dismutase (SOD) 1, SOD2, and catalase, enzymes which are important intracellular anti-oxidant proteins (Carrillo *et al.*, 1991; Carrillo *et al.*, 1992; Carrillo *et al.*, 1993). Recent work has shown that (-)-deprenyl induces the transcription of numerous other antioxidative molecules (such as HO-1, PrxI, TrxI, and TrxRxI) through the activation of NF-E2-related factor-2 nuclear translocation (Nakaso, *et al.*, 2006).

Similarities between the effects of (-)-deprenyl administration after MPTP toxicity to that of administration of a trophic factor (ganglioside GM1 and its internal ester AGF2) after the same insult raise the possibility that (-)-deprenyl may increase trophic support to damaged neurons (Berry, *et al.*, 1994). Axotomy experiments have shown rescue by (-)-deprenyl of motorneurons deprived of muscle-derived trophic support due to the destruction of their striatal axons (Tatton, 1993). Further evidence of trophic support by (-)-deprenyl comes from studies of (-)-deprenyl and nerve growth factor (NGF), which is a neurotrophic factor that promotes survival and growth of CNS and peripheral nerves. (-)-Deprenyl at concentrations lower than those required to inhibit MAO-B increases the synthesis of NGF in cultured cells and intact adult rat cortex cells (Semkova *et al.*, 1996).

Another possible mechanism of action of (-)-deprenyl is an anti-apoptotic effect. In studies of deoxyribonucleic acid (DNA) damage induced by peroxynitrite in cultured cells which do not contain MAO-B (Maruyama and Naoi, 1999), (-)-deprenyl and one of its metabolites, N-desmethyldeprenyl (N-propargylamphetamine), both protected the cells from DNA damage. The activation of anti-apoptotic systems or suppression of pro-apoptotic systems was postulated as the mechanism of action in this study (Maruyama and Naoi, 1999). Pre-incubation with (-)-deprenyl for 20 minutes suppressed DNA damage, implying that new protein synthesis was not required for the protection (Maruyama and Naoi, 1999).

Further support for anti-apoptotic effects is found in work investigating the role of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in programmed cell death. Recent work has shown that GAPDH is an initiator of apoptosis that may be important in both PD and AD (Berry, 2004). Further work demonstrated that (-)-deprenyl and its metabolite N-propargylamphetamine bind to GAPDH and may prevent apoptosis by preventing translocation of GAPDH into the nucleus (Berry, 2004).

In vitro experiments have shown stimulation of sodium, potassium adenosine triphosphatase (Na+, K+-ATPase) by (-)-deprenyl (Antoniades *et al.*, 2002). This transporter is implicated in the uptake and release of catecholamines and neuronal excitability and may be another mechanism for the effects of the drug (Antoniades *et al.*, 2002). The increased Na+,K+-ATPase stimulation may be related to changes in neuronal excitability observed with (-)-deprenyl administration; however these changes may be indirect as stimulation was not observed with pure enzyme (Antoniades, *et al.*, 2002).

A recent study has focused on the role of (-)-deprenyl in cell/cell adhesion, which plays an important role in synaptogenesis and structural plasticity and can affect the survival of neurons (Jenei *et al.*, 2005). (-)-Deprenyl increased irreversible cell-cell adhesion of both neuronal and non-neuronal cells regardless of MAO-B expression (Jenei, *et al.*, 2005). Cell-cell adhesion may help prevent cell death by modulating the effect of α-synuclein (which regulates the activity of the dopamine transporter, and is a major component of Lewy bodies) or by increasing neurogenesis (Jenei, *et al.*, 2005).

(-)-Deprenyl has been reported to show efficacy in improving recovery after strokes in humans (Sivenius *et al.*, 2001), and this effect may be due to its direct action on the mitochondrial membrane potential (Simon *et al.*, 2005). After hypoxia the mitochondrial membrane potential falls, which induces the opening of the mitochondrial permeability transition pores and leads to the release of mitochondrial apoptosis initiation factors (Simon, *et al.*, 2005). (-)-Deprenyl's direct modulation of the mitochondrial membrane potential and thus the respiratory function of the mitochondria may be the basis for some of its anti-apoptotic effects, especially in hypoxia/ischemia models (Simon, *et al.*, 2005).

1.5 (-)-DEPRENYL METABOLISM

(-)-Deprenyl is rapidly absorbed from the gastrointestinal tract, and is 94% plasma protein bound at therapeutic concentrations (Gerlach, *et al.*, 1996). The half-life of (-)-deprenyl is not established. It has been reported as approximately 25 min. (Gerlach, *et al.*, 1996), 90 min. (with up to 4-fold increse with chronic administration (Mahmood, 1997) and 8-10 days (in rats) (Tatton, 1993). (-)-Deprenyl is extensively metabolized in the liver to (-)-methamphetamine and (-)-N-propargylamphetamine (N-desmethyldeprenyl) which are further metabolized to (-)-amphetamine (Figure 1.2) (Am *et al.*, 2004). Early papers identified (-)-methamphetamine and (-)-amphetamine in human urine and showed that less then 1% of the administered (-)-deprenyl dose was excreted as (-)-deprenyl (Reynolds *et al.*, 1978). (-)-Methamphetamine has been shown to be neurotoxic at low concentrations (Am *et al.*, 2004; McCann and Ricaurte, 2004), while N-propargylamphetamine shows some neuroprotective action (Stocchi and Olanow, 2003). No racemic transformation has been detected at (-)-deprenyl's chiral center during metabolism (Gerlach, *et al.*, 1996). Selegeline is prescribed as the (-)-isomer because this isomer is 150 times more potent at inhibiting MAO-B than the (+)-isomer. Further, the metabolites of (+)-deprenyl [(+)-amphetamine and (+)-methamphetamine] are 10 times more potent CNS stimulants than the (-) isomers and are addicting (Gerlach, *et al.*, 1996). The most comprehensive analysis of (-)-deprenyl metabolism in humans also identified minor hydroxylated compounds (Figure 1.3) (Shin, 1997) and another study found minor formation of N-oxide compounds (Katagi *et al.*, 2002). None of these compounds showed

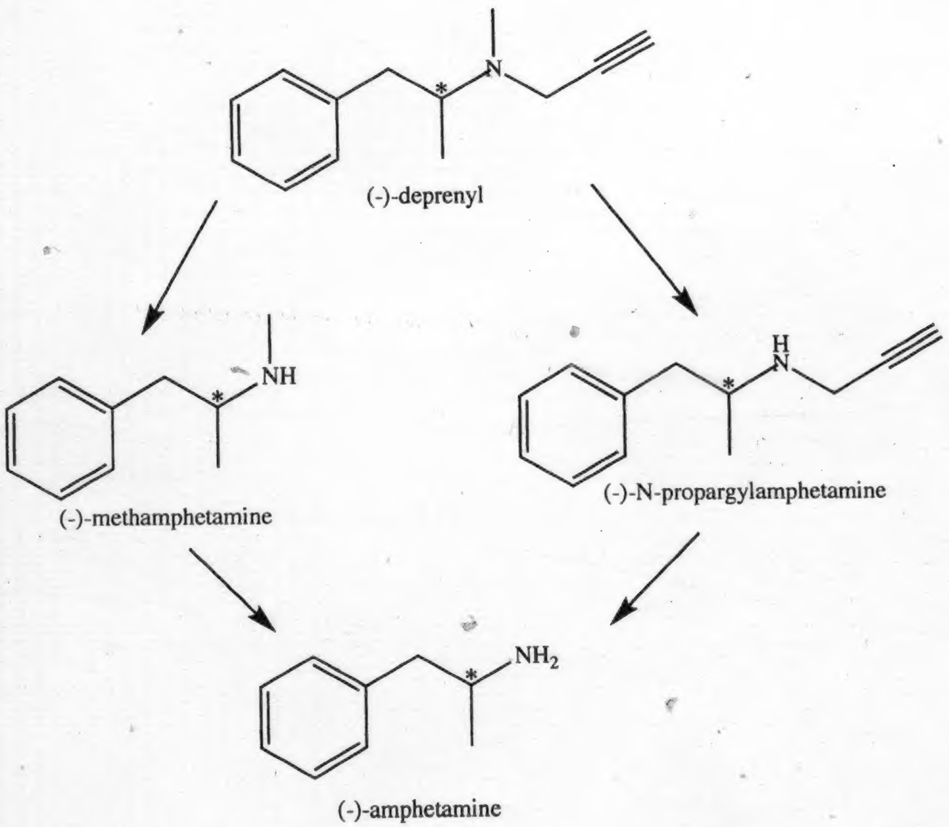
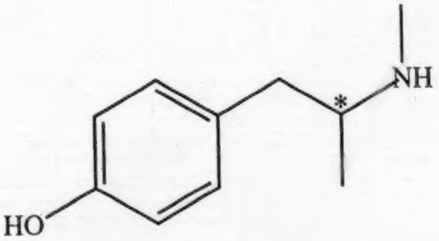
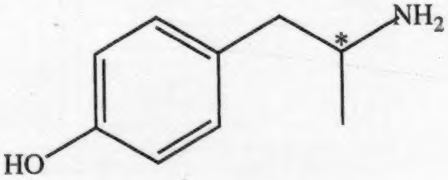

(-)-deprenyl
(-)-methamphetamine
(-)-N-propargylamphetamine
(-)-amphetamine
N
NH
H
N
NH2
*

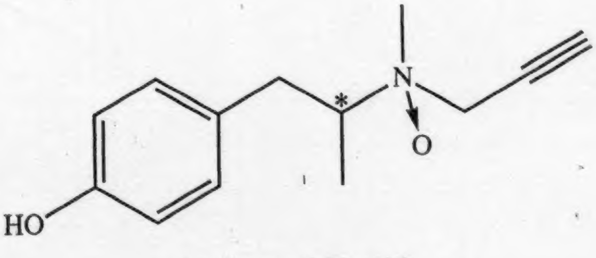
Figure 1.2. Major metabolic routes of deprenyl. These metabolites may be metabolized further by hydroxylation. The chiral centre is indicated by an asterisk.



p-hydroxymethamphetamine



p-hydroxyamphetamine



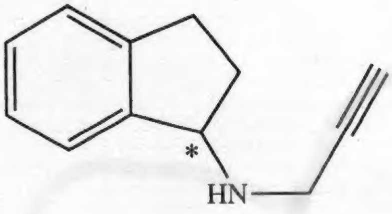
(-)-deprenyl-N-oxide

Figure 1.3. Identified hydroxylated and N-oxide metabolites of deprenyl. The chiral centre is indicated by an asterisk.

chiral switching, although N-oxide forms a second chiral centre and so does form two enantiomers. Early studies showed that amphetamine can be hydroxylated *in vivo* in rat brain tissue (Coutts *et al.*, 1984).

1.6 RASAGILINE

Rasagiline (N-propargyl-1R-aminoindan) (Figure 1.4) is a second generation MAO-B inhibitor that has shown effectiveness in the treatment of PD and has been approved by regulatory agencies in numerous countries, including Canada, for use in PD (Siddiqui and Plosker, 2005). Research on rasagiline was stimulated by the knowledge that (-)-deprenyl, an N-propargylamine, was neuroprotective but that (-)-deprenyl had the disadvantage of being metabolized to the potentially neurotoxic metabolites (-)-amphetamine and (-)-methamphetamine. Rasagiline is an N-propargylamine also but is not metabolized to amphetamines, and its major metabolite, aminoindan, has been reported to be neuroprotective (Am *et al.*, 2004). Rasagiline demonstrated similar potency to (-)-deprenyl *in vitro* in inhibiting MAO-B, but *in vivo* showed greater potency (Siddiqui and Plosker, 2005). Rasagiline also demonstrated neuroprotection in MPTP-induced toxicity and 6-OHDA (6-hydroxydopamine)-induced unilateral lesion models that was not entirely attributable to MAO-B inhibition. This was demonstrated through studies in cells expressing only MAO-A enzyme and by the efficacy of the (S)-isomer of rasagiline, which does not demonstrate MAO-B inhibiting effects (Maruyama *et al.*, 2002). The following effects have also been



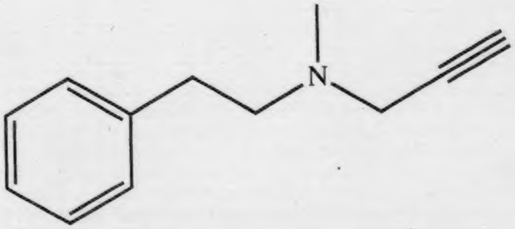
Rasagiline

Figure 1.4. Structure of rasagiline (N-propargyl-1R-aminoindan).

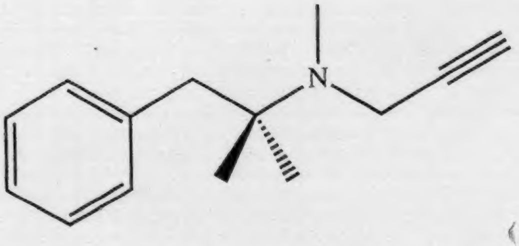
demonstrated with rasagiline: prevention of DNA damage induced by peroxynitrite in human dopaminergic cells (Akao *et al.*, 2002); improvement in neurological severity scores after focal ischemia in rats (Speiser *et al.*, 1999); protection against oxygen-glucose deprivation of PC12 cells (Abu-Raya *et al.*, 1999); and increased neuronal survival of fetal human and rat mesencephalic neurons in culture (Goggi *et al.*, 2000). Rasagiline has also been reported to provide symptom relief in both the early and moderate to late stages of PD (Siddiqui and Plosker, 2005).

1.7 MPPE AND MPPT

N-Methyl,N-propargyl-2-phenylethylamine (MPPE) and N-methyl,N-propargylphentermine (MPPT) are structural analogues of (-)-deprenyl (Figure 1.5) that are neuroprotective in models of neurotoxicity in which (-)-deprenyl has shown efficacy. They were prepared as part of a structure-activity relationship study on possible neuroprotective actions of deprenyl analogues, and are of considerable interest from a metabolic viewpoint as well. If the metabolism of these two compounds parallels that of (-)-deprenyl, the metabolites will not be amphetamine compounds. MPPE would result in formation of N-methyl-2-phenylethylamine (N-methylPEA), N-propargylphenylethylamine (N-propargyl-PEA) and PEA (Figure 1.6) and MPPT would result in N-methylphentermine (N-methylPHEN), N-propargylphentermine (N-propargylPHEN) and phentermine if they paralleled the products of (-)-deprenyl metabolism (Figure 1.7).



N-methyl,N-propargyl-2-phenylethylamine



N-methyl,N-propargylphentermine

Figure 1.5. Structures of N-methyl,N-propargyl-2-phenylethylamine (MPPE) and N-methyl,N-propargylphentermine (MPPT).

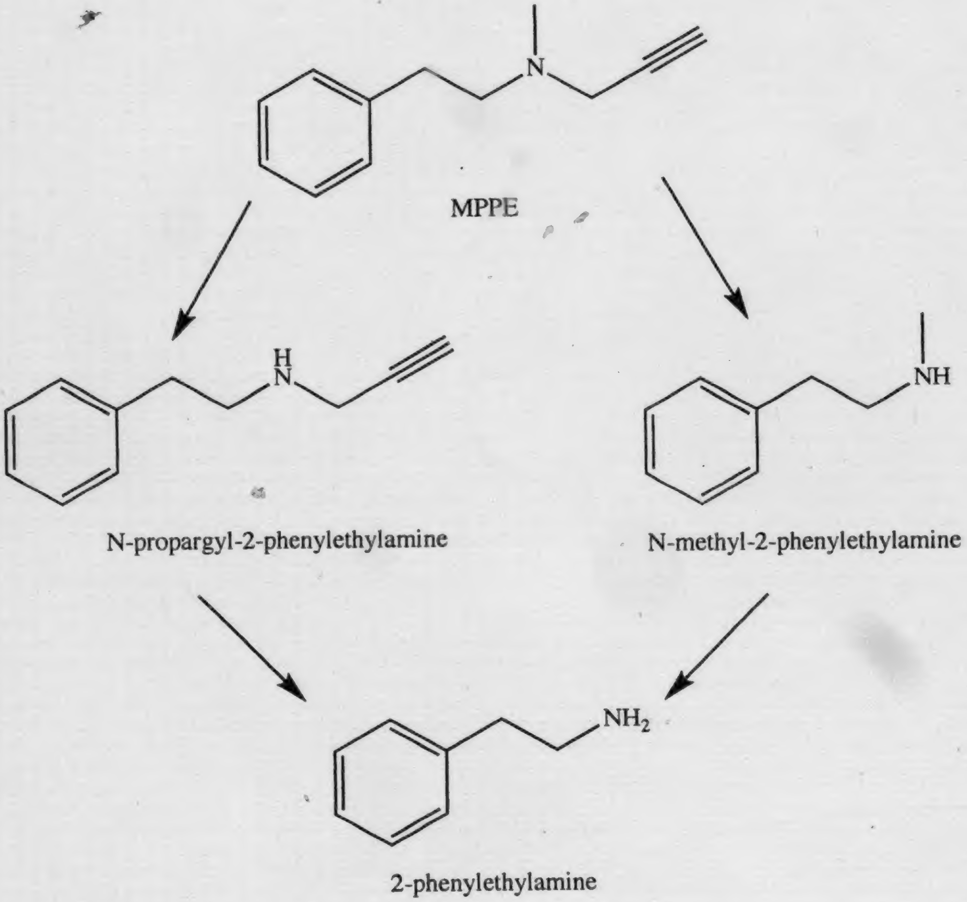

N
MPPE
H
N
NH
N-propargyl-2-phenylethylamine
N-methyl-2-phenylethylamine
NH_2
2-phenylethylamine

Figure 1.6. Metabolism of MPPE to N-methyl-ß-phenylethylamine and N-propargyl-ß-phenylethylamine, further metabolized to ß-phenylethylamine.

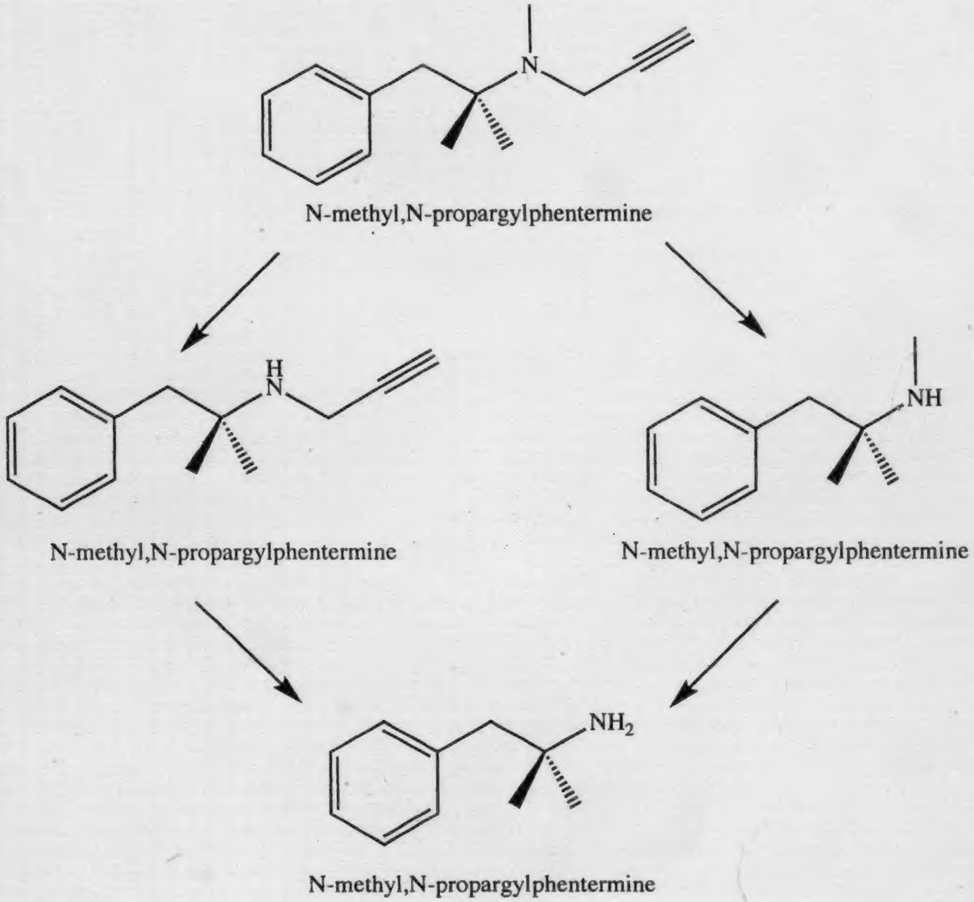

N
N-methyl,N-propargylphentermine
H
N
N-methyl,N-propargylphentermine
NH
N-methyl,N-propargylphentermine
NH_2
N-methyl,N-propargylphentermine

Figure 1.7. Metabolism of MPPT to N-methylphentermine and N-propargylphentermine, further metabolized to phentermine.

The goal of the project described in this thesis was to compare the metabolism of these compounds, by human liver microsomes (HLMs), and to identify the specific enzymes responsible for the metabolic pathways. A considerable amount of time was spent developing assays for the metabolites of MPPE and MPPT and for metabolites of known substrates (i.e. positive controls) of the individual cytochrome P450 (CYP) enzymes investigated.

1.8 XENOBIOTIC METABOLISM

Pharmacologically active lipophilic xenobiotics (non-endogenous compounds that have physiological effects in organisms) will build up in organisms including humans, plants and bacteria, potentially leading to toxic effects. Most of these organisms have systems to chemically decrease the lipophilicity in order to more easily eliminate the exogenous compound, and this process is known as metabolism. In humans, traditionally there are considered to be two major types of xenobiotic metabolism, phase 1 and phase 2. Phase 1 metabolism includes oxidation, hydrolysis and reduction (Brandon *et al.*, 2003) of the xenobiotic. The ultimate effect of these reactions can be hydroxylation, dealkylation, heteroatom oxygenation, or epoxidation of the compound (Wrighton and Stevens, 1992). The CYP family of enzymes is known to catalyze all of these reactions. Phase 2 systems conjugate small endogenous compounds to the xenobiotic to increase water solubility. Examples of Phase 2 reactions are the addition of glucuronides by UDP-glucuronosyltransferase (uridine 5'-diphospho-glucuronosyltransferase) (Miners *et al.*, 2004) and acetates by N-acetyl transferase (Brandon, *et al.*, 2003). Research has also shown an important role for drug transporters, and they have come to be considered a third phase of drug biotransformation (Brandon, *et al.*, 2003). Transporters can affect the duration of drug exposure at the site of metabolism and hence change the exposure and metabolite profile of compounds (Harris *et al.*, 2003). Specifically, multidrug transporters that have a wide range of substrates such as P-glycoprotein, multidrug resistance protein 1 and mitoxantrone resistance protein play an important role in the protection of the body against xenobiotics by eliminating large hydrophobic compounds (Bodo *et al.*, 2003).

Metabolism can occur in stages, i.e. a single compound can be a substrate for both phase 1 and phase 2 metabolism, and in fact can also be metabolized by multiple phase 1 or phase 2 enzymes.

1.8.1 Cytochromes P450

Cytochrome P450 (CYP) enzymes are ubiquitous in nature, found in 23 eukaryotes (both plants and animals) and 6 prokaryotes (Wrighton and Stevens, 1992). There are two classes of CYP enzymes; class 1 enzymes are found primarily in the mitochondrial membranes and class 2 enzymes are bound directly to the endoplasmic reticulum of cells (Kirton *et al.*, 2002). Xenobiotic metabolism is primarily a function of class 2 CYP enzymes. Fifty-seven CYP enzyme genes have been identified in humans (Guengerich, 2006) and can be organized by their primary substrates (Table 1.2). Gene sequencing has led to the systematic and logical grouping of these enzymes based on the similarity of

Sterols	**Xenobiotics**	**Fatty acids**	**Eicosanoids**	**Vitamins**	**Unknown**
1B1	1A1	2J2	4F2	2R1	2A7
7A1	1A2	4A11	2F3	24	2S1
7B1	2A6	2B1	4F8	26A1	2U1
8B1	2A13	4F12	5A1	26B1	2W1
11A1	2B6		8A1	26C1	3A43
11B1	2C8			27B1	4A22
11B2	2C9				4F11
17	2C18				4F22
19	2C19				4V2
21A2	2D6				4X1
27A1	2E1				4Z1
39	2F1				20A1
46	3A4				27C1
51	3A5				
	3A7				

Table 1.2. The 57 human CYP enzymes and their primary substrates [modified from (Guengerich, *et al.*, 2005)].

amino acid sequences. Families share 40% and are identified by an Arabic number (Wrighton and Stevens, 1992) and subfamilies share 70% (Gibson and Skett, 2001) and are identified by a letter. The final Arabic number represents the individual enzyme. For example, CYP 2C19 is the 19th enzyme in the family 2, subfamily C. In humans, the first three families (CYP1, CYP2, and CYP3) are extensively involved in the metabolism of xenobiotics. The entire CYP family of enzymes also plays an important role in the synthesis of many fatty acids, bile acids and steroids and can activate procarcinogens.

In humans, CYP enzymes are found in the highest concentrations in the smooth endoplasmic reticulum of the liver, although they are also found extrahepatically in the mucosa of the intestine, kidneys, lungs, and brain (Lin and Lu, 2001). The enzymes in the mucosa of the intestine contribute to the metabolism of some drugs, and there is increasing evidence that CYP expression in the brain has clinical relevance (Stoffel-Wagner, 2003) . CYP levels in the brain are low in comparison to liver levels, but in individual areas of the brain they can be significantly higher and may affect individual responses to xenobiotics (Miksys and Tyndale, 2002). However, CYP enzymes in the brain probably do not affect overall metabolism and clearance of drugs (Miksys and Tyndale, 2002; Haining, 2007); the site of most metabolism is the liver, and hence the CYP enzymes expressed in the liver are of primary importance in metabolism studies.

1.8.2 CYP structure and mechanism of action

The CYP enzymes are external monooxygenases that catalyze the incorporation of a single oxygen atom into a substrate with the concomitant reduction of the other atom of oxygen to water (Bernhardt, 2006). They contain a heme moiety and a protein component; the heme moiety is the conserved catalytic site, while the protein component varies and results in differing active sites and substrate specificity. The xenobiotic-metabolizing CYPs are unusual for enzymes in that they can catalyze the metabolism of a wide variety of substrates, producing various products.

A catalytic cycle for CYP enzymes was first proposed in 1968 and although it has been updated, it is still considered correct (Denisov *et al.*, 2005). The CYP reaction cycle contains multiple distinct intermediate states and at least three branch points where multiple side reactions are possible, making the system quite complex.

In brief, using hydroxylation as an example, the binding of the substrate to the CYP enzyme displaces a water molecule normally coordinated to the Fe^{3+} (attached through hydrogen bonding instead of covalent bonding). This replacement results in an increase in spin state and corresponding change in reduction potential (Denisov, *et al.*, 2005). An electron is then transferred from nicotinamide adenine dinucleotide phosphate, reduced (NADPH) (the reducing agent) to the iron-substrate complex, reducing the iron molecule (Bernhardt, 2006) and making dioxygen binding energetically favorable (Denisov, *et al.*, 2005). The bound dioxygen accepts the second electron from NADPH, producing a ferric peroxy anion. Protonation of this anion forms the relatively unstable ferric hydroperoxy complex which undergoes heterolytic cleavage to form Compound 1 (Bernhardt, 2006). Compound 1 has not been directly studied due to its rapid conversion to the product complex. The product complex releases the hydroxylated substrate and the original catalytic compound (Figure 1.8).

1.8.3 Factors affecting xenobiotic metabolism

Rates of metabolism can be altered by inhibition, induction and polymorphisms. The mechanisms of CYP inhibition are reversible, quasi- irreversible and irreversible (Lin and Lu, 2001). Both quasi-irreversible and irreversible inhibition inactivate the enzyme and require synthesis of new enzyme to regain catalytic activity (Lin and Lu, 2001). The difference between these two types of inhibition is that quasi-irreversible inhibition is the result of anintermediate metabolite that binds very tightly but reversibly to the enzyme, whereas the metabolite covalently bonds to the enzyme structure in irreversible inhibition.

Reversible inhibition is subdivided into competitive, noncompetitive or uncompetitive (Lin and Lu, 2001). A competitive inhibitior binds to the active site of the CYP enzyme, precluding binding of the substrate. This type of inhibition is common because any time two or more xenobiotics are metabolized by the same enzyme, the one with highest affinity will competitively inhibit metabolism of the others. Noncompetitive inhibition is characterized by the inhibitor binding to a location other than the active site of the enzyme and making the substrate-enzyme-inhibitor complex inactive while not affecting substrate

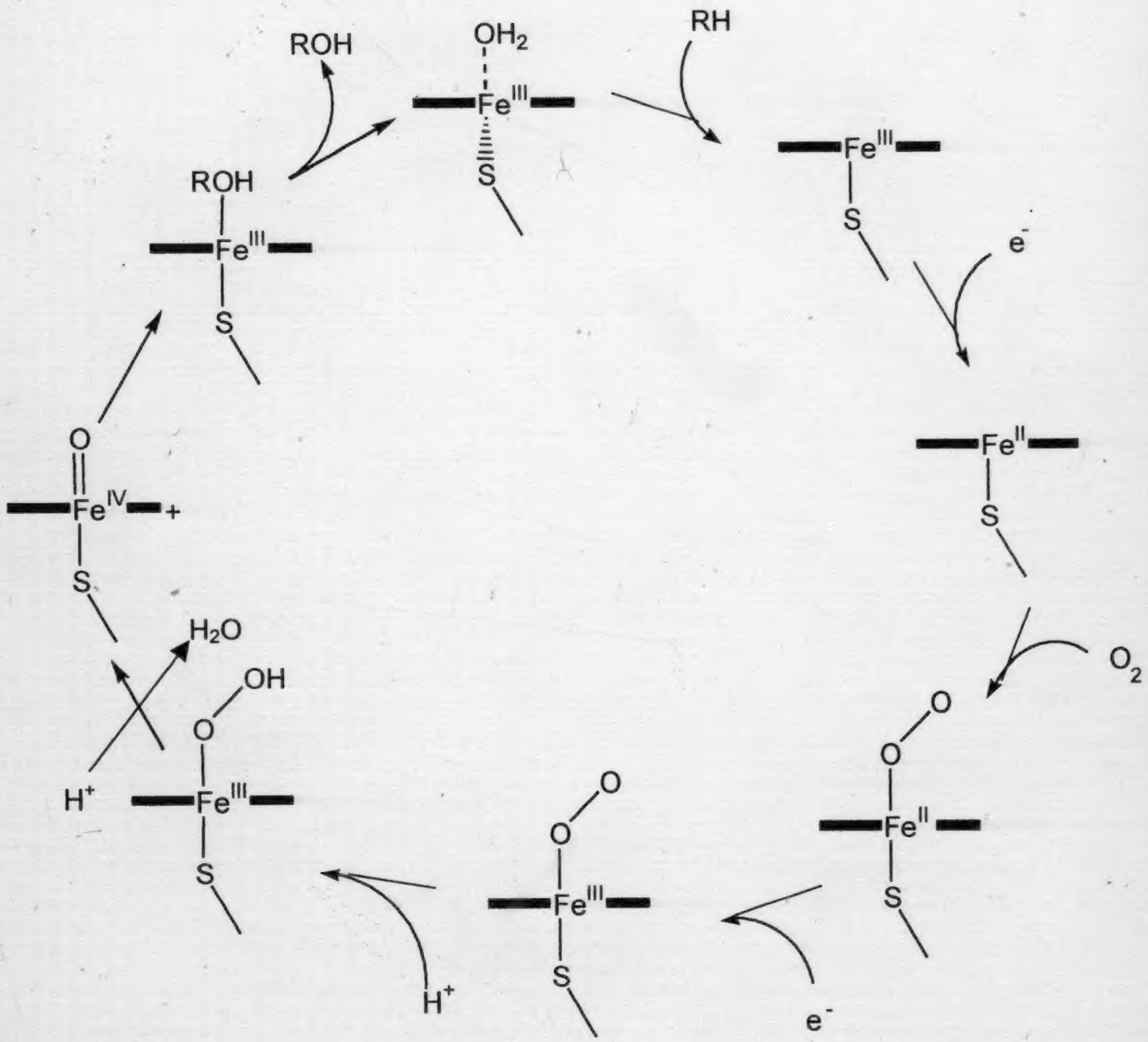
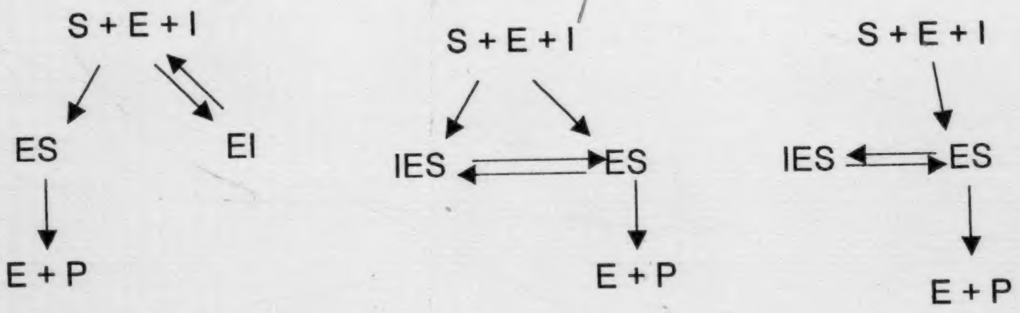

ROH
OH2
RH
Fe^III
S
ROH
Fe^III
S
e-
Fe^II
S
O
Fe^IV
+
S
H2O
OH
O2
O
O
H+
Fe^III
S
O
O
Fe^II
S
O
O
Fe^III
S
H+
e-

Figure 1.8. Simplified CYP P450 catalytic cycle (modified from Denisov, *et al.*, 2005).

binding (Lin and Lu, 2001). In uncompetitive inhibition the inhibitor binds after the substrate has bound to the enzyme and inactives the enzyme-substrate complex (Figure 1.9).

Some of the xenobiotic-metabolizing CYP enzymes are inducible, resulting in increased metabolism rates (Lin and Lu, 2001). Induction is due to increased concentrations of protein through synthesis and so is a slower process than inhibition. The increase in enzyme concentration can be a result of increased transcription of the associated gene, or the result of decreased degradation of the enzyme.

Another factor affecting xenobiotic metabolism rates, polymorphism, is defined as variations in a gene that are present in greater than 1% of the population. Many of the xenobiotic-metabolizing CYP enzymes are polymorphic,many with multiple polymorphisms. In fact, only CYP1A1 and CYP1E1 seem to be conserved. They are theorized to be essential to cell survival due to roles in the cell cycle and gluconeogenesis respectively and hence polymorphisms would potentially be self-limiting (Ingelman-Sundberg, 2001). Most polymorphisms produce less active or non-active forms of the enzymes, resulting in poor metabolizer (PM) phenotypes. The polymorphisms result in altered splicing, stop codons, substrate recognition sites and folding patterns (Ingelman-Sundberg, 2001). There are some documented cases of mutations producing extra copies of the CYP gene (specifically for CYP2D6 and


S + E + I
ES
EI
E + P
S + E + I
IES
ES
E + P
S + E + I
IES
ES
E + P

(1) (2) (3)

Figure 1.9. Competitive (1), noncompetitive (2) and uncompetitive (3) inhibition schematics. S = substrate, E = enzyme, I = inhibitor and P = product.

CYP2C19), which results in extra enzyme and the genotype of ultra-rapid metabolizers (URMs) (Sim *et al.*, 2006).

1.8.4. Individual enzymes of importance in (-)-deprenyl metabolism

1.8.4.1 CYP2B6

CYP2B6 accounts for less than 5% of total CYP P450 in the human liver, which originally led to an underestimation of the contribution of CYP2B6 in human metabolism (Dicke *et al.*, 2005). New estimates are that it metabolizes more than 3% of all clinically used drugs in humans (Shebley *et al.*, 2006). Specifically, it plays a significant role in metabolizing cyclic amines such as phencyclidine and nicotine (Shebley, *et al.*, 2006). CYP2B6 is expressed in the liver, brain, kidney, intestine, lung and heart (Gervot *et al.*, 1999; Thum and Borlak, 2000), although the importance of expression in these organs has not been elucidated. Expression in brain has been demonstrated to be region-specific, mainly neuronal and increased in smokers compared to non-smokers (Miksys and Tyndale, 2002). CYP2B6 is polymorphic, and the variants *4, *5 and *6 account for 28.6, 9.5 and 25% of allelic frequency in healthy white subjects (Lotsch *et al.*, 2006). It has been demonstrated that these polymorphisms have clinical relevance, as the clearance of bupropion (a 2B6 substrate) is higher in carriers of allele *4, without influence of allele *6 (Lotsch, *et al.*, 2006) and 2B6 genotypes alter abstinence rates in buproprion smoking cessation trials (Lee *et al.*, 2007).

1.8.4.2 CYP2C19

CYP2C19 has been identified as the (S)-mephenytoin hydroxylase enzyme that was first identified as polymorphic in 1979. Currently 7 alleles have been identified which result in PM genotypes and show different distributions among different populations (Desta *et al.*, 2002). However, a recently identified variant causes ultra-rapid metabolism by CYP2C19 (Furuta *et al.*, 2005) and perhaps explains some of the patients who do not respond to common dosages of certain antidepressants (Sim, *et al.*, 2006). CYP2C19 polymorphisms have demonstrated clinical relevance to many psychiatric drugs including anticonvulsants and hypnosedatives such as mephenytoin (excessive sedation seen in PMs), diazepam (lower clearance in orientals than caucasions which is linked to a higher prevelance of PMs in oriental populations), and phenobarbital (neurotoxicity has been reported to result when coprescribed with a CYP2C19 inhibitor). Some antidepressants are also metabolized by CYP2C19; examples are the N-demethylation of tertiary amine tricyclic antidepressants and some selective serotonin reuptake inhibitors (Desta, *et al.*, 2002). Clinically relevant changes in plasma levels of these antidepressants have been reported when administered with 2C19 inhibitors (Desta, *et al.*, 2002). This enzyme has also been reported to be inducible *in vivo* by rifampicin (an antibiotic) and artemisinin (a natural product antimalarial drug) (Desta, *et al.*, 2002), which may also have clinical consequences.

1.8.4.3 CYP2D6

CYP2D6 is one of the most commonly studied enzymes in the CYP family because it metabolizes about 25% of all medications (Cascorbi, 2003). More than 73 different haplotypes have been recorded for CYP2D6, but a limited number are commonly found (Cascorbi, 2003). This enzyme also shows decreased and increased activity due to polymorphisms, though PMs are more common than ultrarapid ones. Although this enzyme can be inhibited by several common drugs such as quinidine, fluoxetine, and sertraline, it is not generally inducible (Cascorbi, 2003).

1.8.5 Drug interactions

Adverse drug interactions (ADIs) occur when two or more drugs taken together alter the effectiveness or toxicity of at least one of them (Sikka *et al.*, 2005). The alterations can be pharmacokinetic or pharmacodynamic in nature (Spina *et al.*, 2003). Pharmacokinetic interactions involve changes in the absorption, distribution, metabolism or excretion of the drugs and their metabolites. Pharmacodynamic interactions occur when two drugs have the same or interrelated mechanisms of action, resulting in additive, synergistic or antagonistic effects (Spina, *et al.*, 2003). It is difficult to determine how often ADIs occur as some studies record theoretical interactions along with ones that have clinical effects, which may be why estimates vary between 2.2% and 30% in hospitalized patients and 9.2% and 70.3% in ambulatory patients (Sikka, *et al.*, 2005). Because the CYP family of enzymes is involved in the metabolism of a majority of pharmaceutical agents, and because they can be inhibited and induced by coprescribed medications, they have been studied extensively in the field of ADIs.

Many psychiatric and neurologic diseases are chronic and require long term care and medication, due to side effects and coexisting conditions, multiple drugs are often coadministered. This makes ADIs more likely, and metabolism studies are very important in the fields of psychiatry and neurology.

1.8.5.1 Methods for studying drug interactions

The most relevant metabolism data come from *in vivo* studies using healthy humans. Ideally, these studies would take into account absorption, drug transport, protein binding and both phase 1 and 2 metabolism and give a total profile of metabolites. However, there are many ethical and practical problems with such studies for investigational drugs. In order to overcome these issues, several *in vitro* strategies for studying metabolic pathways and activity have been developed. These include both complex and simple systems. Complex systems attempt to include many aspects of metabolism; for instance, the use of liver slices can reveal both phase 1 and phase 2 metabolic pathways without the exogenous addition of cofactors (Brandon, *et al.*, 2003). Simple systems investigate less complex questions; for example, many phase 1 and phase 2 enzymes can be expressed in transgenic cell lines and investigated individually. The studies on the metabolism of MPPE and MPPT that follow in this thesis use human liver microsomes and complementary deoxyribonucleic acid (cDNA)-expressed individual enzymes, both of which have well established roles when used properly (Salonen *et al.*, 2003).

The human liver microsomes (HLMs) used in the studies described in this thesis are considered a simple drug metabolism system as the data collected are informative about a single component of the drug metabolism process, the CYP family of enzymes. These HLMs are treated in such a way to maximize the amount of active CYP enzyme and minimize the amount of other enzymes isolated from samples of human livers that are generally harvested during biopsies or postmortem. HLMs also contain flavin-containing monooxygenases and UDP-glucuronyltransferases, although the CYP enzymes are of primary interest in the following studies (Venkatakrishnan *et al.*, 2001). After proper preparation by differential centrifugation, isolated segments of smooth endoplasmic reticulum with active CYP systems remain (Brandon, *et al.*, 2003). These preparations can be incubated *in vitro* with necessary co-factors and the drug of interest; the resulting metabolites can then be isolated, identified and measured. As with all experimental procedures, there are advantages and disadvantages inherent in this system. The advantages include: the elimination of ethical and financial responsibilities of using experimental animals; the elimination of complications arising from differing CYP profiles between species; affordability; and availablity. However, this system provides no information about protein binding or drug distribution that takes place in *in vivo* environments, and because the CYP enzymes are enriched and have little competition from other metabolizing enzymes, the results can only be used for quantitative estimations (Brandon, *et al.*, 2003). Another potential disadvantage is the use of non-therapeutic drug concentrations in an effort to increase the concentration of metabolites to simplify analysis (Iwatsubo *et al.*, 1997). Finally, there is great interindividual variability in the concentration and activity of CYP enzymes in humans. This variability can be countered to some extent by the use of pooled microsomes (Brandon, *et al.*, 2003).

Commercially available human cDNA-expressed individual CYP enzymes allow the metabolic contribution of individual enzymes to be studied and specific interactions researched (Brandon, *et al.*, 2003). An advantage of these systems is the relatively high expression of the enzyme, allowing for biotransformation studies of individual enzymes that are not expressed in high amounts in human liver, but which may be of primary importance in the clearance of some drugs (for instance CYP2B6). However, the interpretation of the results can be complicated when multiple enzymes are involved in the metabolic pathway of the drug. Some success has been found by taking into account the amount of enzyme present in averaged human livers (Stormer *et al.*, 2000).

1.8.6 ENZYME KINETICS

The overlapping substrate specificity of CYP enzymes complicates analysis of metabolism data and necessitates the use of the Michaelis-Menten equation (Equation 1.1) to determine the contribution of individual enzymes (Clarke, 1998). The constants V_{max} (the maximum rate of metabolite formation) and K_m (the concentration of substrate that results in an initial velocity that is one

$$v = (V_{max} * [S])/(K_M + [S])$$

Equation 1.1. Michaelis-Menten equation. v = initial reaction velocity at a given substrate concentration ([S]), V_{max} = maximum possible reaction velocity, and K_M = Michaelis constant, the concentration of substrate at which the velocity is ½ of V_{max}.

half of V_{max}) are used for comparisons between different substrates of the same enzyme (Holt, 2006). They can also be used to predict drug-drug interactions and the relative contribution of individual enzymes to the metabolism of the substrate (lower K_m values translate to higher affinity of the substrate for the enzyme). Inhibition of CYP enzymes can also be studied by comparing K_m values after incubation with an inhibitor. One technique for predicting these interactions is a fluorometric screen (Crespi and Stresser, 2000; Miller *et al.*, 2000). cDNA-expressed individual CYP enzymes are incubated with varying concentrations of the drug of interest and a known substrate that produces a fluorescent product upon metabolism by the CYP enzyme. The change in fluorescence produced is proportional to the interaction between the drug under investigation and the enzyme. While this does not determine if the drug is a substrate for the enzyme, it does provide evidence of an interaction which could lead to ADIs.

1.9 ANALYTICAL TECHNIQUES RELEVANT TO THIS THESIS

1.9.1 Gas Chromatography

Chromatography refers to the separation of compounds in a mixture based on physical differences by distribution between a mobile and stationary phase. Gas-liquid chromatography (GC) is a process in which compounds in a sample are separated based on vapor pressures and differences in affinity for the stationary phase (a high boiling point liquid) versus the gaseous mobile phase. The time between sample injection and detection of the individual compound eluting from the column is called the retention time (RT). Compounds that have limited solubility in the stationary phase will exit the column quickly and have a short RT, as a large proportion will remain in the mobile phase. Compounds with similar polarity to the stationary phase will have longer RTs and potentially broader peaks, due to increased interaction with the stationary phase.

A limitation of GC is the fact that compounds must be vaporized before entering the capillary column, so they must be heat-stable. One method commonly used to increase stability of compounds at higher temperatures is derivatization. This technique also increases the sensitivity of some detectors, specifically the electron-capture detector (ECD) in the experiments in this thesis.

1.9.1.1 Instrumentation

Gas chromatographs are composed of an inlet, carrier gas, a column within an oven and a detector (Figure 1.10). There is a variety of inlets, but they all ensure that a representative sample reproducibly, and frequently automatically, reaches the column (Rittenbach and Baker, 2007). In the last 25 years a major shift has occurred in the type of columns used in GC, from the original packed columns to capillary columns. Capillary columns provide greater separation efficiency, but sample size must be limited, which can make detection of analytes present in low concentration more difficult (Watson, 1999). Capillary columns are now generally made from fused silica, for inertness, and coated with polyamide, to provide strength and flexibility. The stationary phase is coated along the inner walls of these columns in thicknesses from 0.1 to 10.0 μm; both the thickness and the polarity of the stationary phase affect separation,

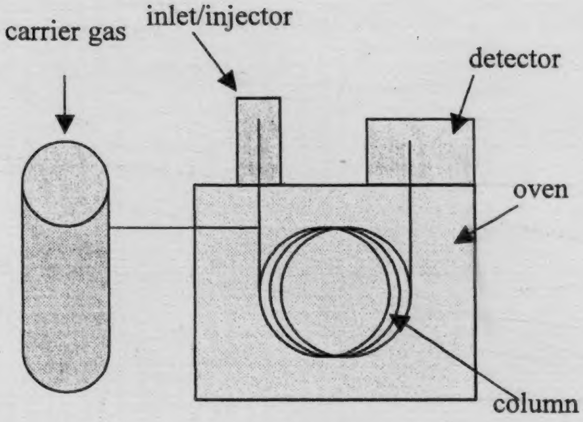

inlet/injector
carrier gas
detector
oven
column

Figure 1.10. Schematic of a gas chromatograph.

and many variations are commercially available. There are two types of capillary columns: wall coated open tubular (WCOT) and support coated open tubular (SCOT) (Coutts and Baker, 1982). A thin liquid phase layer of stationary phase is coated directly onto the walls of WCOT columns. In SCOT columns the thin layer of stationary phase is coated onto a thin layer of support material that coats the walls of the column. WCOT columns can be loaded with larger volumns than SCOT, while SCOT columns have improved stability and allow higher temperatures with little to no breakdown of the stationary phase. The projects in this thesis were all completed using WCOT columns.

Polarity of the column changes RTs dramatically because of the changes in interactions between the compound and the stationary phase. A polar compound will elute faster from a non-polar column than from a polar column because it will be in the mobile phase more in the non-polar column. Usually the temperature of the column is also manipulated to reduce RTs and separate the compounds more completely. This works primarily because increasing the temperature increases the proportion of a compound in the mobile gas phase versus the stationary phase because partition coefficients are temperature-dependent.

There is a variety of detectors commonly used with GC, and the detector used for analysis is usually determined by the compounds being investigated. For the compounds investigated in this thesis, an ECD was used. In this detector a small radioactive source, usually nickel 63, interacts with an appropriate carrier gas (argon-methane in this case) to create a constant stream of free electrons.

This stream of electrons is measured by the collector as the "standing current" (Dybowski and Kaiser, 2002). When an electrophoric substance (substances that attract electrons) exits the column into this stream of electrons, some of the electrons are absorbed by the substance, resulting in a reduction of the standing current, which is recorded as a peak. This detector is very sensitive to halogenated compounds and to compounds containing ketone or nitro groups (Burtis et al., 1987). With technological advances, linearity has been increased since the first development of the ECD, making it useful for many analyses (Coutts and Baker, 1982). For the compounds investigated in this thesis, derivatization with halogenated aromatic moieties was used to increase sensitivity.

1.9.1.2 Derivatization

Derivatization often involves the replacement of an active hydrogen atom by a moiety that improves chromatography and/or sensitivity of the original compound. Improvement of the chromatograph often results from increased volitility of the compound, reduced polarity (which can result in sharper peaks), and/or stabilization of thermally labile structures (Burtis et al., 1987). Derivatization is also used to improve sensitivity of specific GC detectors; for example, adding halogenated moieties can make compounds detectable at much lower concentrations with ECDs. The project described in this thesis used derivatization of activated hydrogens bound to basic nitrogen atoms by moieties that are both aromatic and halogenated (Figure 1.11) to increase sensitivity. The

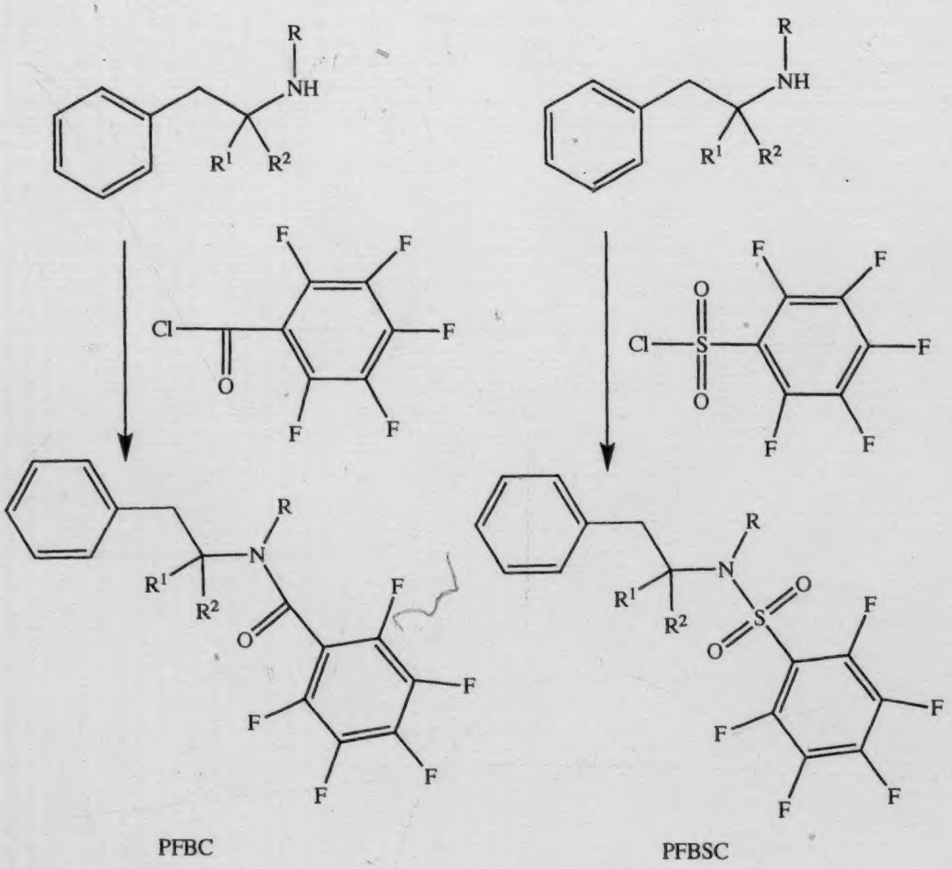

R
NH
R1
R2
F
Cl
O
PFBC
PFBSC
S
N

Figure 1.11. Schematic of derivatization of secondary amines with PFBC and PFBSC. R, R1 and R2 represent hydrogen, methyl or propargyl groups in this thesis.

use of pentafluorobenzenesulfonyl chloride (PFBSC) and pentafluorobenzoyl chloride (PFBC) to derivatize compounds with amine or phenol moieties is well established and can be conducted under aqueous or anhydrous conditions (Delbeke *et al.*, 1983; Sentissi *et al.*, 1984; Baker, G.B. *et al.*, 1986a; Baker, G. B. *et al.*, 1986b; Rao *et al.*, 1986; Coutts *et al.*, 1987; Nazarali *et al.*, 1987; Wong *et al.*, 1988; Paetsch *et al.*, 1992; Salsali *et al.*, 2000; Asghar *et al.*, 2001; Liu *et al.*, 2002)

1.9.2 HIGH PERFORMACE LIQUID CHROMATOGRAPHY

HPLC (high performance liquid chromatography) separates compounds based on differences between affinities for the mobile phase, as in GC, but the mobile phase is a liquid and the stationary phase is coated on small particles in a packed column. The liquid mobile phase can be a variety of solvents including aqueous buffers and organic solvents such as acetonitrile and methanol. The choice is usually dictated by the solubility of the compounds and stability of the stationary phase. When the stationary phase is polar and the mobile phase is non-polar, the least polar compounds (i.e. most nonpolar) will be eluted first; this is considered normal phase chromatography. Generally this stationary phase is a silica gel with hydroxy groups exposed that interact with polar groups of the compounds being analyzed (Watson, 1999). However, many biological compounds are polar, which has led to the common use of reverse-phase chromatography. In this case, the stationary phase is nonpolar and the mobile phase is polar; frequently the stationary phase is silica gel that has been treated so that the hydroxyl groups are replaced with long chain hydrocarbons. The hydrocarbons interact through van der Waals interactions with non-polar compounds (Watson, 1999). In this case the more polar compounds elute first and as the polarity of the mobile phase is decreased, the non-polar compounds are eluted. The advantages of HPLC include that it does not require vaporization of the sample, which simplifies the analysis of molecules with high boiling points and those that are unstable at high temperatures, which include many biological molecules such as high molecular weight proteins. For this reason HPLC has revolutionized analysis of biological molecules.

1.9.2.1 Instrumentation

HPLC instruments are more complicated than gas chomatographs. This is due to the difficulty in maintaining a constant flow of mobile phase when changing the composition during an analytical run. This requires a reservoir for solvents and a precise pumping system in addition to the injector, column, detector and data recorder. The pumping systems have evolved over the decades and now micropumps capable of delivering precise mixes of several liquid phase solvents are commercially available (Watson, 1999). This is vital to HPLC because changes in the percentage of aqueous (polar) to organic (nonpolar) solvents affect retention times for compounds significantly. Columns used for HPLC have a greater diameter but are much shorter than those used in GC and in general are packed with treated silica. Unlike GC, the temperature of the column is not changed during an HPLC run, and in fact caution is taken to maintain a constant temperature. The treatment of the silica creates siloxane linkages that alter the polarity of the stationary phase, affecting retention of compounds. Newer packing materials have emerged that are stable at greater pH ranges, negating one disadvantage of HPLC use which is that silica packing breaks down at both high and low pH levels. Detectors commonly used in HPLC analysis include ultraviolet/visible spectrophotometric (UV/VIS) detectors, fluorescence detectors, electrochemical detectors and mass spectrometers (Odontiadis and Rauw, 2007). In this thesis, the UV/VIS detector was used. This common detector uses a light source with known wavelength (frequently a mercury lamp set at 254 nanometers) and records absorption of this wavelength by compounds as they elute from the column (Odontiadis and Rauw, 2007).

1.9.3 Mass spectrometry

Mass spectrometry (MS) is a very powerful analytical technique that has become readily accessable to many laboratories. There are many types of MS analyzers (for reviews see Sloley *et al.*, 2007), but only the type used in this thesis will be described here. Compounds introduced into an electron ionization mass spectrometer are ionized by a stream of electrons, resulting initially in the production of a high energy molecular ion (the original compound minus one electron). The positively charged ion is drawn into the MS by electric energy, and because it is in a high energy state it fragments into semi-stable derivatives. The fragmentation of compounds is well studied and proceeds through well known patterns, which allows for identification of important structural components of the compounds. Many instruments use a quadrupole to separate ions as they travel to the detector. A quadrupole consists of four parallel rods that create two electrical fields at right angles (Watson, 1999). The electrical fields are produced by direct current in two opposite rods and radiofrequency in the other two (Silverstein *et al.*, 1991). The fields are oscillated, which creates an environment that limits the ions that can pass through the four cylinders to a single weight to charge ratio (m/z). The instrument can scan a range of m/z or single m/z values, increasing sensitivity in the latter case. In the research in this thesis, MS was used to confirm structures of derivatives formed for GC-ECD analysis.

1.9.3.1 Instrumentation

The MS system has an injector (frequently connected directly to a GC or HPLC), an ionization chamber, ion separator (quadrapoles are commonly used in bench top GC analysis), and a data handling system. MS systems must be operated at high vacuums in order to decrease the possibility of the ions colliding with air molecules, which would contaminate the spectra significantly.

1.10 OBJECTIVES AND HYPOTHESIS

The hypotheses investigated in this thesis are that MPPE and MPPT are metabolized by human liver microsomes similarly to (-)-deprenyl, resulting in N-methylPEA, N-propargylPEA, and PEA (MPPE), and N-methylPHEN, N-propargylPHEN and PHEN (MPPT) instead of methamphetamine, N-propargylamphetamine and amphetamine. The objectives are: to establish analytical procedures for the proposed metabolites; determine if they are products of metabolism of MPPE and MPPT by human liver microsomes; and establish which CYP enzymes catalyze the formation of the primary metabolites using a fluorogenic screen. Additional studies will use individual cDNA-expressed CYP enzymes to compare the effects of these CYP enzymes on metabolism of MPPE and MPPT to (-)-deprenyl when the same concentrations of drugs are incubated under the same time conditions. These experiments will utilize human enzymes so that the results will be directly relevant to clinical use.

1.11 REFERENCES

Abu-Raya, S., Blaugrund, E., Trembovler, V., Shilderman-Bloch, E., Shohami, E. and Lazarovici, P. (1999) Rasagiline, a monoamine oxidase-B inhibitor, protects NGF-differentiated PC12 cells against oxygen-glucose deprivation. *J Neurosci Res* 58:456-63

Akao, Y., Maruyama, W., Yi, H., Shamoto-Nagai, M., Youdim, M. B. and Naoi, M. (2002) An anti-Parkinson's disease drug, N-propargyl-1(R)-aminoindan (rasagiline), enhances expression of anti-apoptotic bcl-2 in human dopaminergic SH-SY5Y cells. *Neurosci Lett* 326:105-8

Akhondzadeh, S., Tavakolian, R., Davari-Ashtiani, R., Arabgol, F. and Amini, H. (2003) Selegiline in the treatment of attention deficit hyperactivity disorder in children: a double blind and randomized trial. *Prog Neuro-Psychopharmacol & Biol. Psychiatry* 27:841-5

Alafuzoff, I., Helisalmi, S., Heinonen, E.H., Reinikainen, K., Hallikainen, M., Soininen, H. and Koivisto, K. (2000) Selegiline treatment and the extent of degenerative changes in brain tissue of patients with Alzheimer's disease. *Eur J Clin Pharmacol* 55:815-19

Am, O. B., Amit, T. and Youdim, M. B. H. (2004) Contrasting neuroprotective and neurotoxic actions of respective metabolites of anti-Parkinson drugs rasagiline and selegiline. *Neurosci Lett* 355:169-172

Antoniades, C., Carageorgiou, H. and Tsakiris, S. (2002) Effects of (-)deprenyl (selegiline) on acetylcholinesterase and Na^+,K^+-ATPase activities in adult rat whole brain. *Pharmacol Res* 46:165-9

Asghar, S. J., Baker, G. B., Rauw, G. A. and Silverstone, P. H. (2001) A rapid method of determining amphetamine in plasma samples using pentafluorobenzenesulfonyl chloride and electron-capture gas chromatography. *J Pharmacol Toxicol* 46:111-5

Baker, G. B., Koilpillai, M., Nazarali, A. J., Rao, T. S. and Coutts, R. T. (1986a) Gas chromatography of antidepressants and their metabolites as pentafluorobenzoyl and pentafluorobenzenesulfonyl derivatives. *Proc West Pharmacol Soc* 29:291-294

Baker, G. B., Rao, T. S. and Coutts, R. T. (1986b) Electron-capture gas chromatographic analysis of beta-phenylethylamine in tissues and body fluids using pentafluorobenzenesulfonyl chloride for derivatization. *J Chromatogra-Biomed Appl* 381:211-7

Bartzokis, G., Beckson, M., Newton, T., Mandelkern, M., Mintz, J., Foster, J. A., Ling, W. and Bridge, T. P. (1999) Selegiline effects on cocaine-induced changes in medial temporal lobe metabolism and subjective ratings of euphoria. *Neuropsychopharmacol* 20:582-90

Bernhardt, R. (2006) Cytochromes P450 as versatile biocatalysts. *J Biotechnol* 124:128-45

Berry, M. D. (2004) Glyceraldehyde-3-phosphate dehydrogenase as a target for small-molecule disease-modifying therapies in human neurodegenerative disorders. *J Psychiatr Neurosci* 29:337-45

Berry, M. D., Juorio, A. V. and Paterson, I. A. (1994) Possible mechanisms of action of (-)-deprenyl and other MAO-B inhibitors in some neurologic and psychiatric disorders. *Prog Neurobiol* 44:141-61

Birkmayer, W., Knoll, J., Riederer, P., Youdim, M. B., Hars, V. and Marton, J. (1985) Increased life expectancy resulting from addition of L-deprenyl to Madopar treatment in Parkinson's disease: a longterm study. *J Neural Transm* 64:113-27

Bodkin, J. A., Siris, S. G., Bermanzohn, P. C., Hennen, J. and Cole, J. O. (2005) Double-blind, placebo-controlled, multicenter trial of selegiline augmentation of antipsychotic medication to treat negative symptoms in outpatients with schizophrenia. *Am J Psychiatry* 162:388-390

Bodo, A., Bakos, E., Szeri, F., Varadi, A. and Sarkadi, B. (2003) The role of multidrug transporters in drug availability, metabolism and toxicity. *Toxicology letters* 140-141:133-43

Boix, F., Qiao, S. W., Kolpus, T. and Sagvolden, T. (1998) Chronic L-deprenyl treatment alters brain monoamine levels and reduces impulsiveness in an animal model of Attention-Deficit/Hyperactivity Disorder. *Behav Brain Res* 94:153-62

Brandon, E. F., Raap, C. D., Meijerman, I., Beijnen, J. H. and Schellens, J. H. (2003) An update on in vitro test methods in human hepatic drug biotransformation research: pros and cons. *Toxicol Appl Pharmacol* 189:233-46

Burtis, C.A., Bowers, L.D., Chattoraj, S.C., and Ullman, M.D., 1987, Chromatography, in: Fundamentals of Clinical Chemistry 3rd edition, N.W. Tietz. ed. W.B. Saunders Company, Philadelphia pp. 105-123.

Carrillo, M. C., Kanai, S., Nokubo, M. and Kitani, K. (1991) (-)-Deprenyl induces activities of both superoxide dismutase and catalase but not of glutathione peroxidase in the striatum of young male rats. *Life Sci* 48:517-21

Carrillo, M. C., Kanai, S., Sato, Y., Nokubo, M., Ivy, G. O. and Kitani, K. (1993) The optimal dosage of (-)deprenyl for increasing superoxide dismutase activities in several brain regions decreases with age in male Fischer 344 rats. *Life Sci* 52:1925-34

Carrillo, M. C., Kitani, K., Kanai, S., Sato, Y. and Ivy, G. O. (1992) The ability of (-)deprenyl to increase superoxide dismutase activities in the rat is tissue and brain region selective. *Life Sci* 50:1985-92

Cascorbi, I. (2003) Pharmacogenetics of cytochrome p4502D6: genetic background and clinical implication. *Eur J Clin Invest* 33 Suppl 2:17-22

Clarke, S. E. (1998) In vitro assessment of human cytochrome P450. *Xenobiotica* 28:1167-202

Coutts, R. T. and Baker, G. B. (1982) Gas Chromatography in Handbook of Neurochemistry, 2nd edition. Volume 2: Experimental Neurochemistry. A.Lajtha ed. Plenum Press, New York pp. 429-448.

Coutts, R.T., Prelusky, D.B. and Baker, G.B. (1984) Determination of amphetamine, norephedrine and their phenolic metabolites in rat brain by gas chromatography. *J Pharm Sci.* 73:808-812

Coutts, R. T., Torok-Both, G. A., Tam, Y. K., Chu, L. V. and Pasutto, F. M. (1987) Acetylation and pentafluorobenzoylation of lidocaine metabolites in aqueous solution and identification of derivatives by combined gas chromatography/mass spectrometry. *Biomed Environ Mass Spectrom* 14:173-82

Crespi, C. L. and Stresser, D. M. (2000) Fluorometric screening for metabolism-based drug-drug interactions. *J Pharmacol Toxicol Methods* 44:325-31

Delbeke, F. T., Debackere, M., Jonckheere, J. A. and De Leenheer, A. P. (1983) Pentafluorobenzoyl derivatives of doping agents. I. Extractive benzoylation and gas chromatography with electron-capture detection of primary and secondary amines. *J Chromatogr* 273:141-9

Denisov, I. G., Makris, T. M., Sligar, S. G. and Schlichting, I. (2005) Structure and chemistry of cytochrome P450. *Chem Rev* 105:2253-77

Desta, Z., Zhao, X., Shin, J. G. and Flockhart, D. A. (2002) Clinical significance of the cytochrome P450 2C19 genetic polymorphism. *Clin Pharmacokinet* 41:913-58

Dicke, K. E., Skrlin, S. M. and Murphy, S. E. (2005) Nicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-butanone metabolism by cytochrome P450 2B6. *Drug Metab Dispos* 33:1760-4

Dybowski, C. and Kaiser, M.A., (2002), Chromatography in Kirk-Othmer Encyclopedia of Chemical Technology, John Wiley and Sons, Inc; http://www.mrw.interscience.wiley.com/kirk/articles/chrokais.a01/frame.html.

Ebadi, M., Sharma, S., Shavali, S. and El Refaey, H. (2002) Neuroprotective actions of selegiline. *J Neurosci Res* 67:285-9

Filip, V. and Kolibas, E. fo the Czech and Slovak Senile Dementia of Alzheimer Type Study Group (1999) Selegiline in the treatment of Alzheimer's disease: a long-term randomized placebo-controlled trial. *J Psychiat Neursci* 24:234-43

Finnegan, K. T., Skratt, J. J., Irwin, I., DeLanney, L. E. and Langston, J. W. (1990) Protection against DSP-4-induced neurotoxicity by deprenyl is not related to its inhibition of MAO B. *Eur J Pharmacol* 184:119-26

Fohey, K. D., Hieber, R. and Nelson, L. A. (2007) The role of selegiline in the treatment of negative symptoms associated with schizophrenia. *Ann Pharmacother* 41:851-6

Fritschy, J. M. and Grzanna, R. (1991) Selective effects of DSP-4 on locus coeruleus axons: are there pharmacologically different types of noradrenergic axons in the central nervous system? *Progr Brain Res* 88:257-68

Furuta, T., Sagehashi, Y., Shirai, N., Sugimoto, M., Nakamura, A., Kodaira, M., Kenmotsu, K., Nagano, M., Egashira, T., Ueda, K., Yoneyama, M., Ohashi, K., Ishizaki, T. and Hishida, A. (2005) Influence of CYP2C19 polymorphism and Helicobacter pylori genotype determined from gastric tissue samples on response to triple therapy for H pylori infection. *Clin Gastroenterol Hepatol* 3:564-73

Gerlach, M., Youdim, M. B. and Riederer, P. (1996) Pharmacology of selegiline. *Neurol* 47:S137-45

Gervot, L., Rochat, B., Gautier, J. C., Bohnenstengel, F., Kroemer, H., de Berardinis, V., Martin, H., Beaune, P. and de Waziers, I. (1999) Human

CYP2B6: expression, inducibility and catalytic activities. *Pharmacogenetics* 9:295-306

Gibson, C. J. (1987) Inhibition of MAO B, but not MAO A, blocks DSP-4 toxicity on central NE neurons. *Eur J Pharmacol* 141:135-8

Gibson, C. J. and Skett (2001) Introduction to Drug Metabolism. Cheltenham, UK, Nelson Thornes Publishers

Goggi, J., Theofilopoulos, S., Riaz, S. S., Jauniaux, E., Stern, G. M. and Bradford, H. F. (2000) The neuronal survival effects of rasagiline and deprenyl on fetal human and rat ventral mesencephalic neurones in culture. *Neuroreport* 11:3937-41

Grossberg, G. T. (2003) Diagnosis and treatment of Alzheimer's disease. *J Clin Psychiatry* 64 Suppl 9:3-6

Guengerich, F. P. (2006) Cytochrome P450s and other enzymes in drug metabolism and toxicity. *AAPS J* 8:E101-11

Guttman, M., Kish, S. J. and Furukawa, Y. (2003) Current concepts in the diagnosis and management of Parkinson's disease. *CMAJ* 168:293-301

Haining, R. L. (2007) Cytochrome P450 reactions in the human brain in Handbook of Neurochemistry and Molecular Neurobiology, 3rd edtion, Practical Neurochemistry Methods, Baker, G.B., Dunn, S.M., and Holt, A. eds. New York, Kluwer Academic / Plenum

Harris, R. Z., Jang, G. R. and Tsunoda, S. (2003) Dietary effects on drug metabolism and transport. *Clin Pharmacokinet* 42:1071-88

Hill, P. (2006) What is neuroprotection? *Medical News Today* Oct 9, 2006. URL: http://www.medicalnewstoday.com/articles/53700.php

Holt, A. (2006) Practical Enzymology: Quantifying enzyme activity and the effects of drugs thereupon, in Handbook of Neurochemistry and Molecular Neurobiology,3rd Edition, Practical Neurochemistry: Methods, Baker, G.B., Dunn, S.M. and Holt, A. eds. New York, Kluwer Academic / Plenum

Houtsmuller, E. J., Notes, L. D., Newton, T., van Sluis, N., Chiang, N., Elkashef, A. and Bigelow, G. E. (2004) Transdermal selegiline and intravenous cocaine: safety and interactions. *Psychopharmacology (Berl)* 172:31-40

Ingelman-Sundberg, M. (2001) Implications of polymorphic cytochrome P450-dependent drug metabolism for drug development. *Drug Metab Dispos* 29:570-3

Iwatsubo, T., Hirota, N., Ooie, T., Suzuki, H., Shimada, N., Chiba, K., Ishizaki, T., Green, C. E., Tyson, C. A. and Sugiyama, Y. (1997) Prediction of in vivo drug metabolism in the human liver from in vitro metabolism data. *Pharmacol Ther* 73:147-71

Jenei, V., Zor, K., Magyar, K. and Jakus, J. (2005) Increased cell-cell adhesion, a novel effect of R-(-)-deprenyl. *J Neural Transm* 112:1433-45

Katagi, M., Tatsuno, M., Tsutsumi, H., Miki, A., Kamata, T., Nishioka, H., Nakajima, K., Nishikawa, M. and Tsuchihashi, H. (2002) Urinary excretion of selegiline N-oxide, a new indicator for selegiline administration in man. *Xenobiotica* 32:823-31

Kirton, S. B., Baxter, C. A. and Sutcliffe, M. J. (2002) Comparative modelling of cytochromes P450. *Adv Drug Deliv Rev* 54:385-406

Knoll, J. and Magyar, K. (1972) Some puzzling pharmacological effects of monoamine oxidase inhibitors. *Adva Biochem Psychopharmacol* 5:393-408

Kosten, T. R., George, T. P. and Kosten, T. A. (2002) The potential of dopamine agonists in drug addiction. *Expert Opinion Invest Drugs* 11:491-9

Lee, A. M., Jepson, C., Hoffmann, E., Epstein, L., Hawk, L. W., Lerman, C. and Tyndale, R. F. (2007) CYP2B6 genotype alters abstinence rates in a bupropion smoking cessation trial. *Biol Psychiat* (in press)

Lin, J. H. and Lu, A. Y. (2001) Interindividual variability in inhibition and induction of cytochrome P450 enzymes. *Annu Rev Pharmacol Toxicol* 41:535-67

Liu, Z. Q., Tan, Z. R., Wang, D., Huang, S. L., Wang, L. S. and Zhou, H. H. (2002) Simultaneous determination of fluoxetine and its metabolite p-trifluoromethylphenol in human liver microsomes using a gas chromatographic-electron-capture detection procedure. *J Chromatogr B* 769:305-11

Lotsch, J., Skarke, C., Wieting, J., Oertel, B. G., Schmidt, H., Brockmoller, J. and Geisslinger, G. (2006) Modulation of the central nervous effects of levomethadone by genetic polymorphisms potentially affecting its metabolism, distribution, and drug action. *Clin Pharmacol Ther* 79:72-89

Magyar, K. and Szende, B. (2004) (-)-Deprenyl, a selective MAO-B inhibitor, with apoptotic and anti-apoptotic properties. *NeuroToxicol* 25:233-242

Magyar, K., Vizi, E. S., Ecseri, Z. and Knoll, J. (1967) Comparative pharmacological analysis of the optical isomers of phenyl-isopropyl-methyl-propinylamine (E-250). *Acta Physiolo Academiae Scientiarum Hungaricae* 32:377-87

Mahmood, I. (1997) Clinical pharmacokinetics and pharmacodynamics of selegiline. An update. *Clin Pharmacokinet.* 33:91-102

Markey, S. P., Johannessen, J. N., Chiueh, C. C., Burns, R. S. and Herkenham, M. A. (1984) Intraneuronal generation of a pyridinium metabolite may cause drug-induced parkinsonism. *Nature* 311:464-7

Maruyama, W. and Naoi, M. (1999) Neuroprotection by (-)-deprenyl and related compounds. *Mech Ageing Dev* 111:189-200

Maruyama, W., Takahashi, T., Youdim, M. and Naoi, M. (2002) The anti-Parkinson drug, rasagiline, prevents apoptotic DNA damage induced by peroxynitrite in human dopaminergic neuroblastoma SH-SY5Y cells. *J Neural Transm* 109:467-81

McCann, U. D. and Ricaurte, G. A. (2004) Amphetamine neurotoxicity: accomplishments and remaining challenges. *Neurosci Biobehav Rev* 27:821-826

Miksys, S. L. and Tyndale, R. F. (2002) Drug-metabolizing cytochrome P450s in the brain. *J Psychiatry Neurosci* 27:406-15

Miller, V. P., Stresser, D. M., Blanchard, A. P., Turner, S. and Crespi, C. L. (2000) Fluorometric high-throughput screening for inhibitors of cytochrome P450. *Ann N Y Acad Sci* 919:26-32

Miners, J. O., Smith, P. A., Sorich, M. J., McKinnon, R. A. and Mackenzie, P. I. (2004) Predicting human drug glucuronidation parameters: application of in vitro and in silico modeling approaches. *Annu Rev Pharmacol Toxicol* 44:1-25

Murphy, B. P., Chung, Y. C., Park, T. W. and McGorry, P. D. (2006) Pharmacological treatment of primary negative symptoms in schizophrenia: a systematic review. *Schizophr Res* 88:5-25

Nakaso, K., Nakamura, C., Sato, H., Imamura, K., Takeshima, T. and Nakashima, K. (2006) Novel cytoprotective mechanism of anti-parkinsonian drug deprenyl: PI3K and Nrf2-derived induction of antioxidative proteins. *Biochem Biophys Res Commun* 339:915-22

Nazarali, A. J., Baker, G. B., Coutts, R. T., Yeung, J. M. and Rao, T. S. (1987) Rapid analysis of beta-phenylethylamine in tissues and body fluids utilizing pentafluorobenzoylation followed by electron-capture gas chromatography. *Prog Neuropsychopharmacol Biol Psychiatry* 11:251-8

Newton, T. F., Kalechstein, A., Beckson, M., Bartzokis, G., Bridge, T. P. and Ling, W. (1999) Effects of selegiline pretreatment on response to experimental cocaine administration. *Psychiatry Res* 87:101-6

Odontiadis, J. and Rauw, G. (2007) High-performance liquid chromatographic analysis of psychotropic and endogenous compounds, in Handbook of Neurochemistry and Molecular Neurobiology,3rd Edition, Practical Neurochemistry: Methods, Baker, G.B., Dunn, S.M. and Holt, A. eds. New York, Kluwer Academic / Plenum

Olanow, C. W. (2004) The scientific basis for the current treatment of Parkinson's disease. *Ann Rev Med* 55:41-60

Ono, K., Hasegawa, K., Naiki, H. and Yamada, M. (2006) Anti-Parkinsonian agents have anti-amyloidogenic activity for Alzheimer's beta-amyloid fibrils in vitro. *Neurochem Int* 48:275-85

Paetsch, P. R., Baker, G. B., Caffaro, L. E., Greenshaw, A. J., Rauw, G. A. and Coutts, R. T. (1992) Electron-capture gas chromatographic procedure for simultaneous determination of amphetamine and N-methylamphetamine. *J Chromatogr-Biomed Appl* 573:313-7

Paolini, M., Sapone, A. and Gonzalez, F. J. (2004) Parkinson's disease, pesticides and individual vulnerability. *Trends Pharmacol Sci* 25:124-129

Patkar, A. A., Pae, C. U. and Masand, P. S. (2006) Transdermal selegiline: the new generation of monoamine oxidase inhibitors. *CNS Spectr* 11:363-75

Rao, T. S., Baker, G. B. and Coutts, R. T. (1986) Pentafluorobenzenesulfonyl chloride as a sensitive reagent for the rapid gas chromatographic analysis of tranylcypromine in tissues and body fluids. *Biochem Pharmacol* 35:1925-8

Reynolds, G.P., Elsworth, J.D., Blau, K., Sandler, M., Lees, A.J. and Stern, G.M. (1978) Deprenyl is metabolized to methamphetamine and amphetamine in man. *Br J Clin Pharmac.* 6:542-4

Riederer, P. and Lachenmayer, L. (2003) Selegiline's neuroprotective capacity revisited. *J Neural Transm* 110:1273-8

Rittenbach, K. and Baker, G. B. (2007) Gas Chromatography, in Handbook of Neurochemistry and Molecular Neurobiology,3rd Edition, Practical Neurochemistry: Methods, Baker, G.B., Dunn, S.M. and Holt, A. eds. New York, Kluwer Academic / Plenum

Salonen, J. S., Nyman, L., Boobis, A. R., Edwards, R. J., Watts, P., Lake, B. G., Price, R. J., Renwick, A. B., Gomez-Lechon, M.-J., Castell, J. V., Ingelman-Sundberg, M., Hidestrand, M., Guillouzo, A., Corcos, L., Goldfarb, P. S., Lewis, D. F. V., Taavitsainen, P. and Pelkonen, O. (2003) Comparative studies on the cytochrome P450-associated metabolism and interaction potential of selegiline between human liver-derived in vitro systems. *Drug Metab Dispos* 31:1093-1102

Salsali, M., Coutts, R. T. and Baker, G. B. (2000) A rapid electron-capture gas chromatographic procedure for the analysis of p-hydroxymephenytoin. *J Pharmacol Toxicol Methods* 44:461-5

Sano, M. (2003) Noncholinergic treatment options for Alzheimer's disease. *J Clin Psychiatry* 64 Suppl 9:23-8

Sano, M., Ernesto, C., Thomas, R. G., Klauber, M. R., Schafer, K., Grundman, M., Woodbury, P., Growdon, J., Cotman, C. W., Pfeiffer, E., Schneider, L. S. and Thal, L. J. (1997) A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimer's disease. The Alzheimer's Disease Cooperative Study. *N Engl J Med* 336:1216-22

Schapira, A. H. V. and Olanow, C. W. (2004) Neuroprotection in Parkinson disease: Mysteries, myths, and misconceptions. *J Am Med Assoc* 291:358-364

Semkova, I., Wolz, P., Schilling, M. and Krieglstein, J. (1996) Selegiline enhances NGF synthesis and protects central nervous system neurons from excitotoxic and ischemic damage. *Eur J Pharmacol* 315:19-30

Sentissi, A., Joppich, M., O'Connell, K., Nazareth, A. and Giese, R. W. (1984) Pentafluorobenzenesulfonyl chloride: a new electrophoric derivatizing reagent with application to tyrosyl peptide determination by gas chromatography with electron capture detection. *Anal Chem* 56:2512-7

Shebley, M., Jushchyshyn, M. I. and Hollenberg, P. F. (2006) Selective pathways for the metabolism of phencyclidine by cytochrome p450 2b enzymes: identification of electrophilic metabolites, glutathione, and N-acetyl cysteine adducts. *Drug Metab Dispos* 34:375-83

Shin, H.-S. (1997) Metabolism of selegiline in humans. Identification, excretion, and stereochemistry of urine metabolites. *Drug Metab Dispos* 25:657-662

Shults, C. W. (2003) Treatments of Parkinson disease: circa 2003. *Arch Neurol* 60:1680-1684

Siddiqui, M. A. and Plosker, G. L. (2005) Rasagiline. *Drugs & Aging* 22:83-91; discussion 93-4

Sikka, R., Magauran, B., Ulrich, A. and Shannon, M. (2005) Bench to bedside: Pharmacogenomics, adverse drug interactions, and the cytochrome P450 system. *Acad Emerg Med* 12:1227-35

Silverstein, R. M., Gassler, G. C. and Morrill, T. C. (1991) Spectrometric identification of organic compounds. New York, John Wiley & Sons, Inc.

Sim, S. C., Risinger, C., Dahl, M. L., Aklillu, E., Christensen, M., Bertilsson, L. and Ingelman-Sundberg, M. (2006) A common novel CYP2C19 gene variant causes ultrarapid drug metabolism relevant for the drug response to proton pump inhibitors and antidepressants. *Clin Pharmacol Ther* 79:103-13

Simon, L., Szilagyi, G., Bori, Z., Telek, G., Magyar, K. and Nagy, Z. (2005) Low dose (-)deprenyl is cytoprotective: it maintains mitochondrial membrane potential and eliminates oxygen radicals. *Life Sci* 78:225-31

Sivenius, J., Sarasoja, T., Aaltonen, H., Heinonen, E., Kilkku, O. and Reinikainen, K. (2001) Selegiline treatment facilitates recovery after stroke. *Neurorehabil Neural Repair* 15:183-90

Sloley, B. D., Rauw, G. and Coutts, R. T. (2007) The analyses of neurotransmitters, other neuroactive substances and their metabolites using mass-spectrometry in Handbook of Neurochemistry and Molecular Neurobiology, 3rd Edition, Practical Neurochemistry Methods, Baker, G.B., Dunn, S.M. and Holt, A. eds. New York, Kluwer Academic / Plenum

Sowa, B. N., Todd, K. G., Tanay, V. A. M. I., Holt, A. and Baker, G. B. (2004) Amine oxidase inhibitors and development of neuroprotective drugs. *Curr Neuropharmacol* 2:153-168

Speiser, Z., Mayk, A., Eliash, S. and Cohen, S. (1999) Studies with rasagiline, a MAO-B inhibitor, in experimental focal ischemia in the rat. *J Neural Transm* 106:593-606

Spina, E., Scordo, M. G. and D'Arrigo, C. (2003) Metabolic drug interactions with new psychotropic agents. *Fundam Clin Pharmacol* 17:517-38

Stocchi, F. and Olanow, C. W. (2003) Neuroprotection in Parkinson's disease: clinical trials. *Ann Neurol* 53 Suppl 3:S87-97; discussion S97-9

Stoffel-Wagner, B. (2003) Neurosteroid biosynthesis in the human brain and its clinical implications. *Ann N Y Acad Sci* 1007:64-78

Stormer, E., von Moltke, L. L. and Greenblatt, D. J. (2000) Scaling drug biotransformation data from cDNA-expressed cytochrome P-450 to human liver: a comparison of relative activity factors and human liver abundance in studies of mirtazapine metabolism. *J Pharmacol Exp Ther* 295:793-801

Takahata, K., Katsuki, H., Kobayashi, Y., Muraoka, S., Yoneda, F., Kume, T., Kashii, S., Honda, Y. and Akaike, A. (2003) Protective effects of selegiline and desmethylselegiline against N-methyl-D-aspartate-induced rat retinal damage. *Eur J Pharmacol* 458:81-9

Tatton, W. G. (1993) Selegiline can mediate neuronal rescue rather than neuronal protection. *Movement Disord* 8 Suppl 1:S20-30

Thomas, T. (2000) Monoamine oxidase-B inhibitors in the treatment of Alzheimer's disease. *Neurobiol Aging* 21:343-348

Thum, T. and Borlak, J. (2000) Gene expression in distinct regions of the heart. *Lancet* 355:979-83

Todd, K. G. and Butterworth, R. F. (1998) Increased neuronal cell survival after L-deprenyl treatment in experimental thiamine deficiency. *J Neurosci Res* 52:240-6

Venkatakrishnan, K., Von Moltke, L. L. and Greenblatt, D. J. (2001) Human drug metabolism and the cytochromes P450: application and relevance of in vitro models. *J Clin Pharmacol* 41:1149-79

Watson, D. G. (1999) Pharmaceutical Analysis: A textbook for pharmacy students and pharmaceutical chemists. Edinburgh, Churchill Livingstone

Wong, J. T., Baker, G. B. and Coutts, R. T. (1988) Rapid and simple procedure for the determination of urinary phenylacetic acid using derivatization in aqueous medium followed by electron-capture gas chromatography. *J Chromatogr* 428:140-6

Wrighton, S. A. and Stevens, J. C. (1992) The human hepatic Cytochromes P450 involved in drug metabolism. *Crit Rev Toxicol* 22:1-21

Yasar, S., Gaal, J., Panlilio, L. V., Justinova, Z., Molnar, S. V., Redhi, G. H. and Schindler, C. W. (2006) A comparison of drug-seeking behavior maintained by D-amphetamine, L-deprenyl (selegiline), and D-deprenyl under a second-order schedule in squirrel monkeys. *Psychopharmacology (Berl)* 183:413-21

Youdim, M. B., Edmondson, D. and Tipton, K. F. (2006) The therapeutic potential of monoamine oxidase inhibitors. *Nature Rev* 7:295-309

Yu, P. H., Davis, B. A., Fang, J. and Boulton, A. A. (1994) Neuroprotective effects of some monoamine oxidase-B inhibitors against DSP-4-induced noradrenaline depletion in the mouse hippocampus. *J Neurochem* 63:1820-8

Zhang, X., Zuo, D. M. and Yu, P. H. (1995) Neuroprotection by R(-)-deprenyl and N-2-hexyl-N-methylpropargylamine on DSP-4, a neurotoxin, induced degeneration of noradrenergic neurons in the rat locus coeruleus. *Neurosci Lett* 186:45-8

CHAPTER 2

A RAPID, SENSITIVE ELECTRON-CAPTURE GAS CHROMATAGRAPHIC PROCEDURE FOR ANALYSIS OF METABOLITES OF N-METHYL,N-PROPARGYLPHENYLETHYLAMINE, A POTENTIAL NEUROPROTECTIVE AGENT.[1]

---

[1] A version of this chapter has been published. Rittenbach, K.A., Sloley, B.D., Ling, L., Coutts, R.T., Shan, J. and Baker, G.B (2005) *Journal of Pharmacological and Toxicological Methods*: 52: 373-378.

2.1 INTRODUCTION

(-)-Deprenyl (N-methyl,N-propargylamphetamine) is a selective, irreversible monoamine oxidase (MAO)-B inhibitor (although at high concentrations MAO-A is also inhibited). It has been reported to have neuroprotective and/or neurorescue properties in a wide variety of *in vivo* and *in vitro* models (Tatton, 1993; Semkova et al., 1996; Tatton and Chalmers-Redman, 1996; Paterson et al., 1998; Tatton et al., 2000; Ebadi et al., 2002; Riederer and Lachenmayer, 2003; Magyar and Szende, 2004; Sowa et al., 2004) and in many cases the mechanisms involved seem to be independent of its effects on MAO-B. (-)-Deprenyl is used clinically to delay motor symptoms in Parkinson's disease (PD) and may be effective at delaying deterioration in Alzheimer's disease (AD). Studies investigating efficacy in PD are extensive and generally show positive results (Heinonen and Myllyla, 1998; Mandel et al., 2003; Stocchi and Olanow, 2003). Improvement in mental cognition, behavior and mood in AD patients have been reported with (-)-deprenyl (DeLaGarza, 2003), but a large meta-analysis by Birks and Flicker (2003) determined that there was no evidence of clinically meaningful benefit, and thus these authors did not recommend use.

A major potential drawback of (-)-deprenyl therapy is the extensive formation of (-)-methamphetamine and (-)-amphetamine as major metabolites (Am et al., 2004), although the other major metabolite, N-propargylamphetamine (Figure 2.1), may be partially responsible for neuroprotective action (Stocchi and Olanow, 2003).

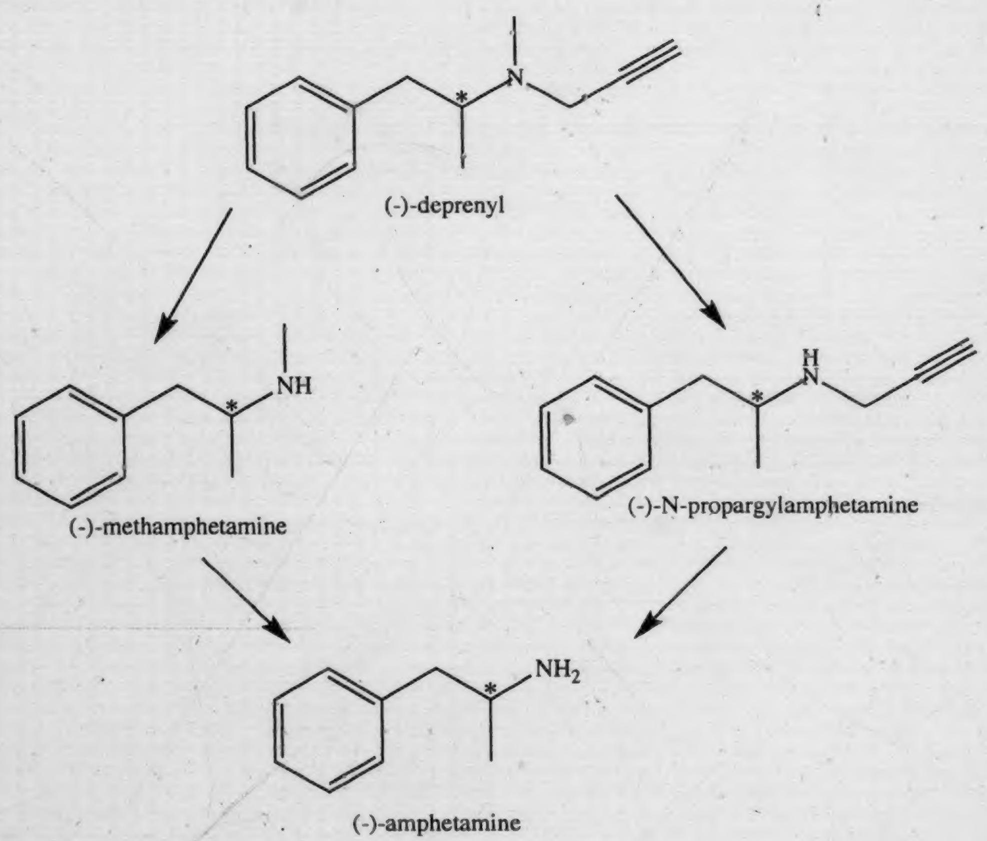

(-)-deprenyl
(-)-methamphetamine
(-)-N-propargylamphetamine
(-)-amphetamine

Figure 2.1. Metabolism of deprenyl to methamphetamine, N-propargylamphetamine and amphetamine.

N-Methyl,N-propargylphenylethylamine (MPPE) is a novel analogue of deprenyl that is also a MAO inhibitor and, like (-)-deprenyl, has been shown to be neuroprotective in the thiamine-deficiency (Kwan et al., 2000) and DSP-4 (Ling *et al.*, unpublished) models of neurotoxicity. Because of its similarity to deprenyl, MPPE is hypothesized to be metabolized to β-phenylethylamine (PEA), N-methylphenylethylamine (N-methylPEA) and N-propargylphenylethylamine (N-propargylPEA) (Figure 2.2). In order to investigate the metabolism of MPPE, a rapid, sensitive method of analysis for the three metabolites was developed in our laboratories. The procedure, which involves derivatization under aqueous conditions with pentafluorobenzenesulfonyl chloride (PFBSC) followed by analysis using gas-chromatography with electron capture detection (GC-ECD), is described below and has been applied to metabolism studies using human liver microsomes.

Derivatization with PFBSC under aqueous conditions is a well established technique for the analysis of endogenous compounds and xenobiotics containing phenol and/or amine groups (Sentissi et al., 1984; Baker et al., 1986a and 1986b; Nazarali *et al.*, 1987; Urichuk et al., 1997; Liu et al., 2002). The reaction with amines is illustrated in Figure 2.3.

2.2 METHODS

2.2.1 Materials

ß-Phenylethylamine (PEA) HCl, N-methylPEA, benzylamine HCl [internal standard (IS)], glucose-6-phosphate, and β-nicotine adenine dinucleotide

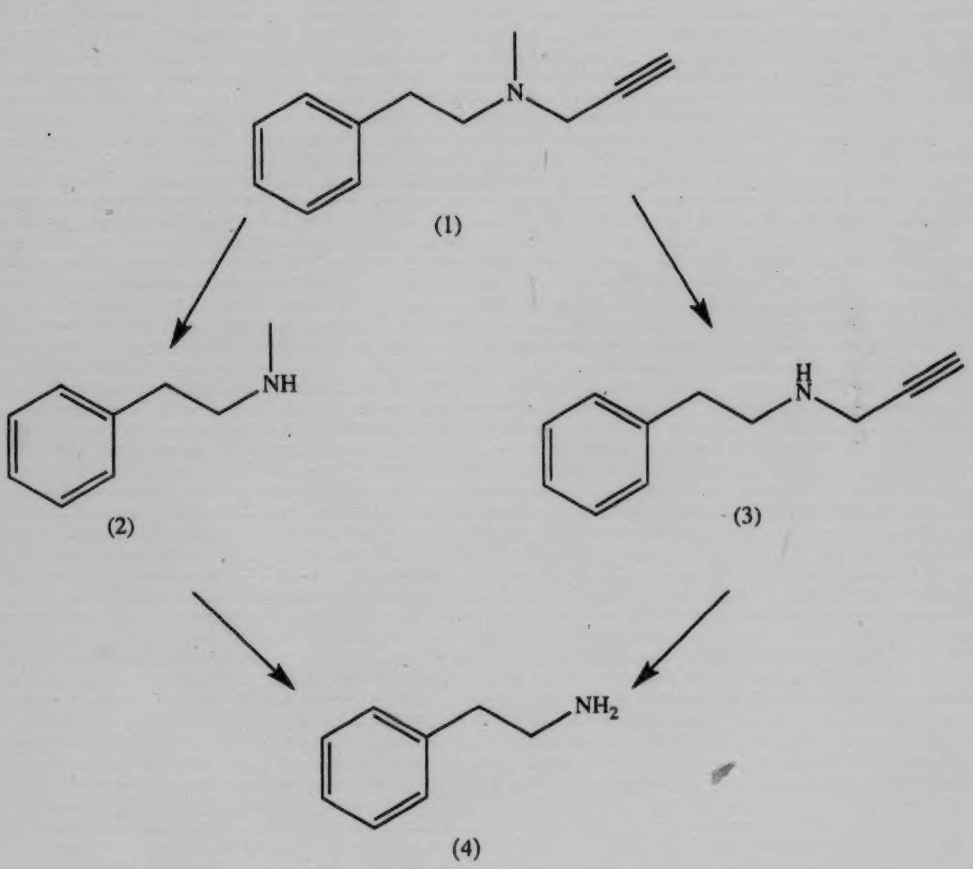


Figure 2.2. Proposed metabolism of MPPE (1) to N-methylphenylethylamine (2), N-propargylphenylethylamine (3) and β-phenylethylamine (4).

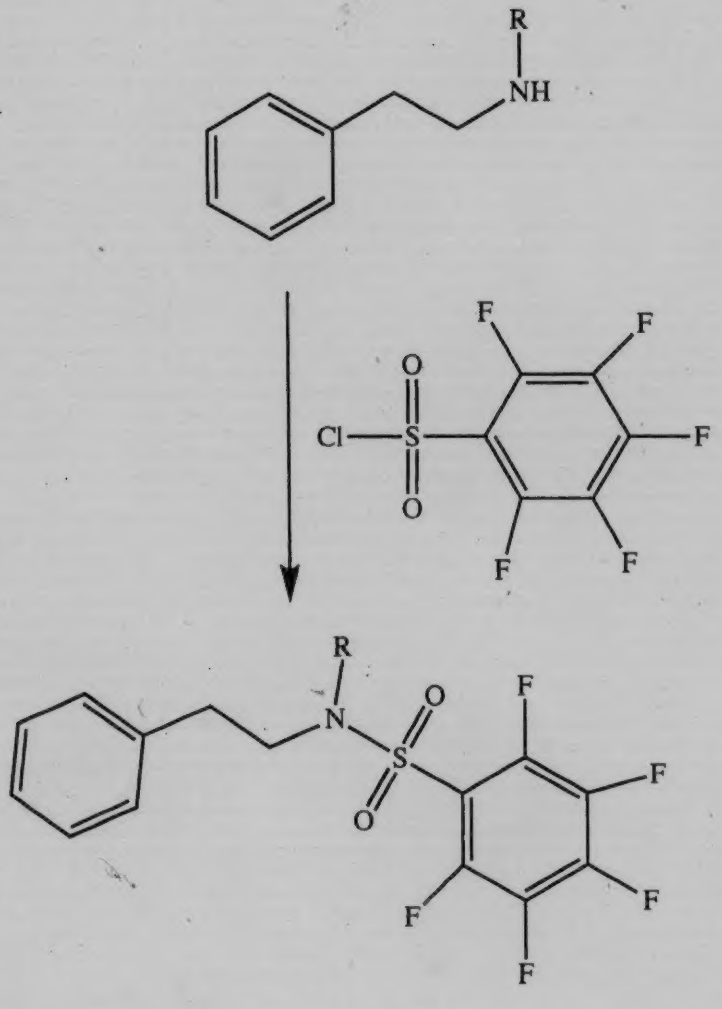


Figure 2.3: Representation of derivatization of phenylethylamines with PFBSC (R=H, CH_3 or CH_2CCH).

phosphate were purchased from Sigma Chemical Company (St. Louis, MO, USA). Glucose-6-phosphate dehydrogenase was purchased from Cederlane Laboratories Limited (Hornby, ON, Canada). MPPE has been previously synthesized in the laboratories of CV Technologies (Edmonton, AB, Canada). For the current study, N-PropargylPEA HCl was synthesized by Dr. E.E. Knaus in the University of Alberta Faculty of Pharmacy and Pharmaceutical Sciences (Edmonton, AB, Canada). The structure of N-propargylPEA was confirmed by nuclear magnetic resonance spectroscopy using a Bruker AM-300 spectrometer. N-propargylPEA was shown to be stable in aqueous solution at 0-4°C for several days, but fresh dilutions were made from frozen stock solutions for each assay run.

The human liver microsome solution (HG-74) was purchased from Gentest Corporation (Woburn, MA, USA). PFBSC was purchased from Sigma Aldrich Canada Ltd (Oakville, ON, Canada).

Ethyl acetate, acetonitrile, toluene, potassium carbonate, potassium bicarbonate, potassium phosphate monobasic, potassium phosphate dibasic and magnesium chloride were purchased from Fisher Scientific (Mississauga, ON, Canada); all were HPLC or reagent grade and were used without further purification.

2.2.2 Human Liver Microsome Incubations

The microsome incubation medium consisted of 25 μl of an NADPH-generating system (4 mg/ml β-nicotine adenine dinucleotide phosphate, 4 mg/ml glucose-6-phosphate, 3.2 units/ml of glucose-6-phosphate dehydrogenase and 2.6 mg/ml of magnesium chloride in 0.1M potassium phosphate buffer (pH=7.4)), 10 µl of microsomal solution, between 5 µl and 25 µl of MPPE substrate solution (resulting in final concentrations of 30-3000 µM) and sufficient buffer to result in a volume of 100 µl. The metabolic reaction was allowed to proceed for 5-60 min in a 37°C water bath. The samples were placed on ice and 50 µl of 25% potassium carbonate solution were added to stop the reaction. Double-distilled water (850 µl) and 40 µl of working IS solution (400 ng) were added and the samples were transferred to screwtop tubes.

2.2.3 Sample Preparation, Derivatization and Analysis

To 1 ml aqueous samples, either prepared standards or human liver microsome samples, an excess of solid potassium bicarbonate was added. The sample was vortexed briefly and allowed to settle. The liquid was decanted into a second screwtop tube and 4 ml of derivatizing reagent (9ml:1ml:10µl; ethyl acetate:acetonitrile:PFBSC) were added. After 2 min of vortex mixing, the mixture was centrifuged at 2500 rpm for 5 min. The upper layer was transferred to a concentration tube and taken to dryness in a Savant Speed-vac evaporator. Toluene (300 µl) was added to the residue and a 2 µl aliquot of this solution was injected for GC-ECD analysis.

Standard curves consisting of a fixed amount of IS (400 ng) and varying amounts of the three analytes of interest were run in parallel with each assay run. Ratios of analytes to IS were plotted on the x axis against amounts of analyte on the y axis. Amounts of analytes in each sample were determined by comparing ratios of analyte to IS in the sample to those in the standard curve.

2.2.4 GC-ECD analysis

The analysis was carried out using a Hewlett-Packard HP-5890A gas chromatograph with an ECD with a Ni-63 source. The GC column used was a HP-5 capillary column (25m x 0.32mm i.d., 1.05μm film thickness). The injection temperature was 250°C. The following column temperature ramp was programmed: initial temperature of 105°C (held for 0.5 min), increasing at 20°C/min to 210°C (held for 5 min), increasing at 2°C/min to 240°C, and increasing at 30°C/min to 295°C (held for 5 min). The carrier gas was helium, with argon-methane (90-10) used as the makeup gas at the detector.

2.2.5 GC-MS analysis

GC-MS analysis was performed on an Agilent 6890 Series gas chromatograph coupled to an Agilent 5973 Network Mass Selective Detector. Analysis was conducted in the electron-impact (electron ionization, EI) mode.

2.3 RESULTS

Initial standard curves prepared with boiled (inactivated) human liver microsomes were compared to standard curves prepared in water, and no significant difference was seen. For example, mean ratios in water versus inactivated human liver microsomes for the standard curves over a range of 9 points were 102.3% for N-methylPEA and 106.6% for PEA. Thereafter, for the sake of economy, all standard curves were prepared in water.

The procedure is rapid and gives peaks with excellent chromatographic properties (Figure 2.4). Structures of the derivatives were confirmed by GC-MS, and the mass fragmentation patterns are shown in Figures 2.5-2.7. The recovery, intra-assay reliability and inter-assay reliability of the method were determined (Table 2.1). Recovery was measured as the percent of a given concentration of analyte extracted from the aqueous medium compared to the same concentration derivatized in the organic phase without extraction. Recoveries were 92.7%, 102.7% and 83.1% for PEA, N-methylPEA and N-propargylPEA, respectively. Intra-assay reliability was calculated by extracting 5 samples of the same concentration (150 ng/ml PEA, 350 ng/ml N-methylPEA or N-propargylPEA) and comparing the analyte to IS ratio. Both PEA and N-methylPEA intra-assay reliabilities were less than 5%, and N-propargylPEA was 8.5%. Interassay reliability was calculated using 150 ng/ml PEA and 400 ng/ml N-methylPEA and N-propargylPEA from 5 curves from different days and values were 1.0%, 2.3% and 2.5% respectively. These results showed that this method is robust and reliable.

The method is linear, with correlation coefficients for standard curves (PEA 5 ng/ml to 250 ng/ml; N-methylPEA 5 ng/ml to 1400 ng/ml; N-propargylPEA 50 ng/ml to 1400 ng/ml) of $r^2>0.99$ obtained routinely. Standard curves usually contained 7-9 points. Typical curves are shown in Figure 2.8. The lowest detectable quantity of the method is 1.5 ng/ml for PEA and <10 ng/ml

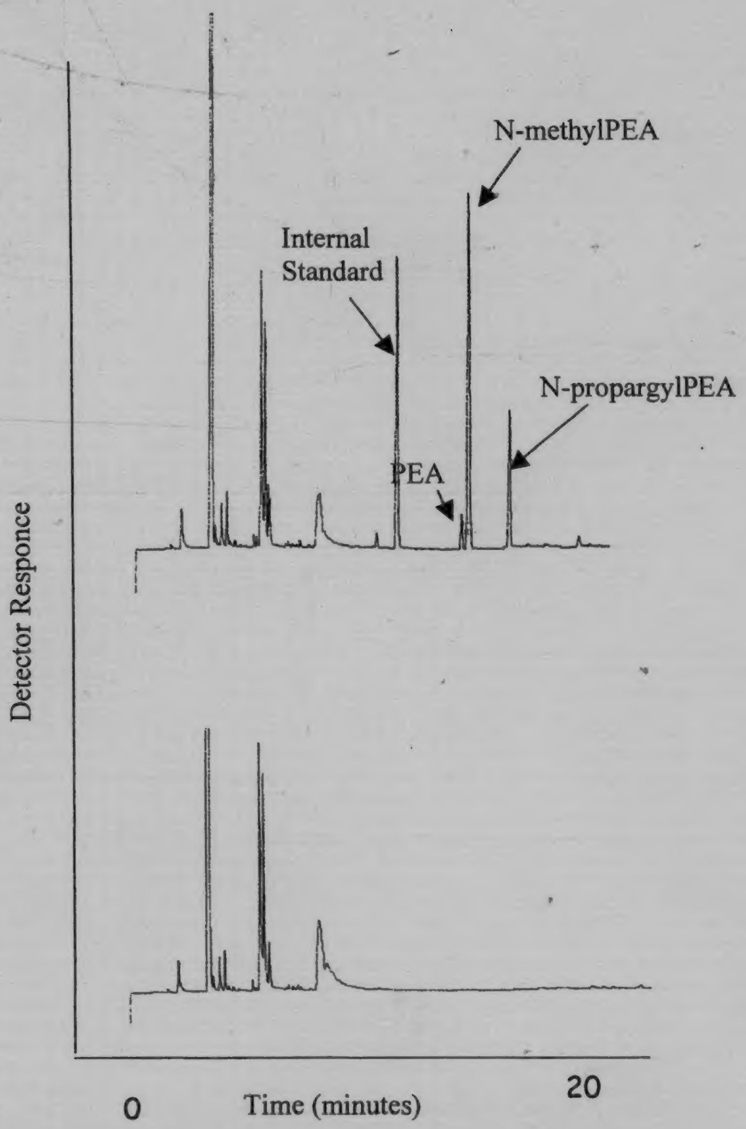


Figure 2.4. Top: example of typical derivatized extract of human liver microsomes incubated for 60 minutes with MPPE (500 uM). Bottom: Corresponding derivatized extract from human liver microsome incubation without MPPE present. The retention times are as follows: IS, 12.0 min; PEA, 15.0 min; N-methylPEA, 15.3 min; and N-propargylPEA, 17.2 min.

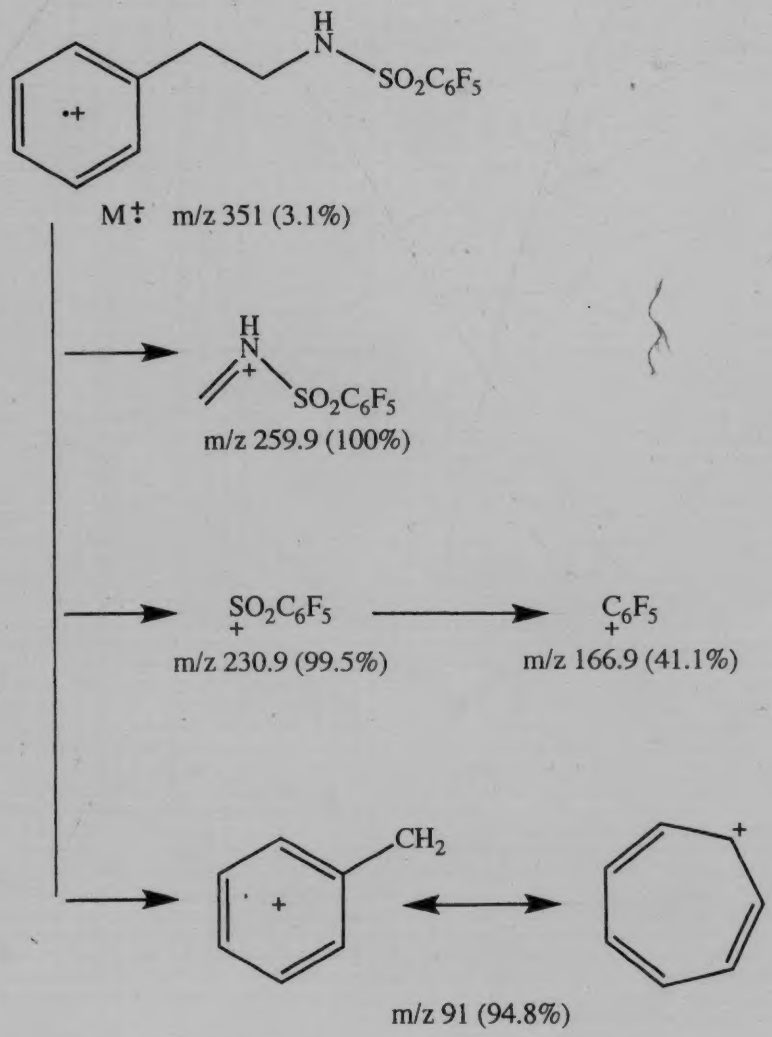

H
N
$SO_2C_6F_5$
•+
M+• m/z 351 (3.1%)
H
N
+
$SO_2C_6F_5$
m/z 259.9 (100%)
$SO_2C_6F_5$
+
m/z 230.9 (99.5%)
C_6F_5
+
m/z 166.9 (41.1%)
CH_2
+
+
m/z 91 (94.8%)

Figure 2.5. Mass spectral fragmentation of β-phenylethylamine derivatized with PFBSC. Percentages represent percent abundance relative to the base peak.

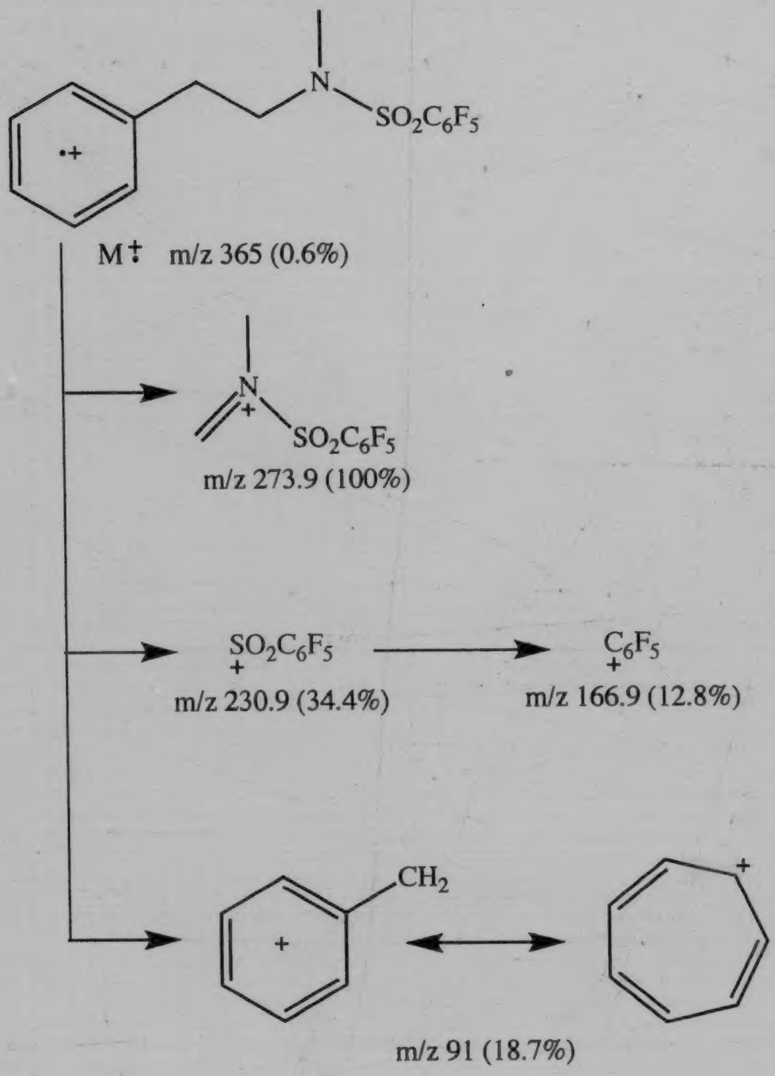

N
SO2C6F5
•+
M+• m/z 365 (0.6%)
N
+
SO2C6F5
m/z 273.9 (100%)
SO2C6F5
+
m/z 230.9 (34.4%)
C6F5
+
m/z 166.9 (12.8%)
CH2
+
+
m/z 91 (18.7%)

Figure 2.6. Mass spectral fragmentation of N-methylphenylethylamine derivatized with PFBSC. Percentages represent percent abundance relative to the base peak.

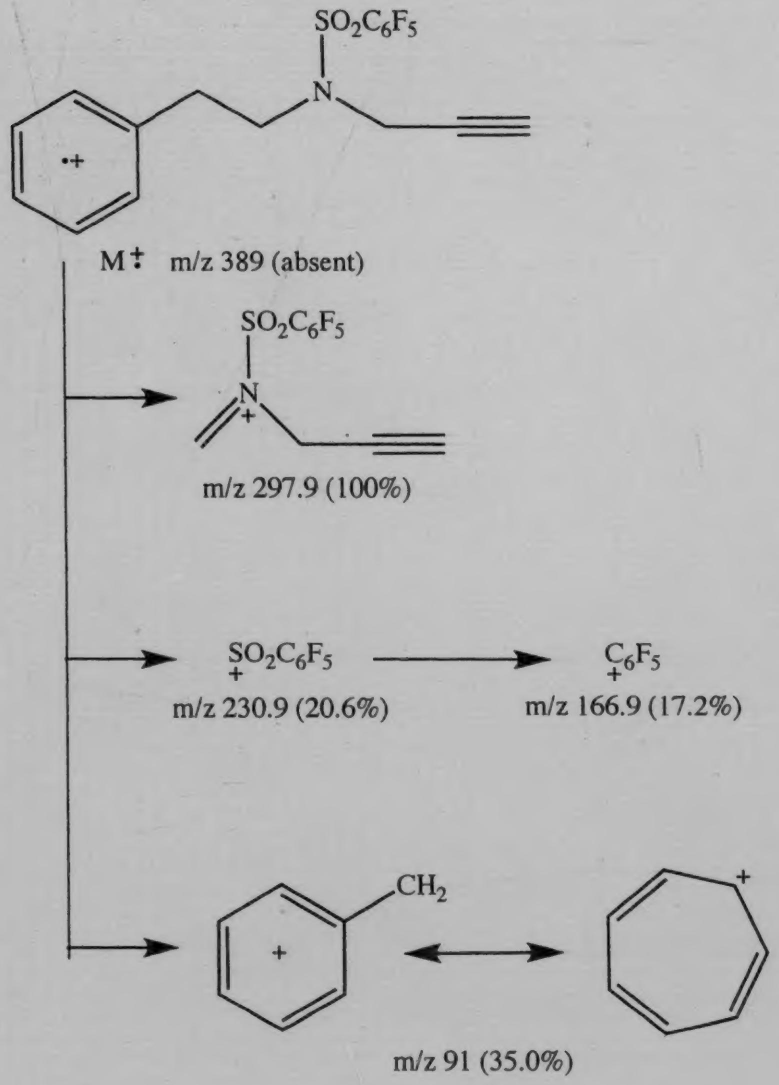

SO2C6F5
N
•+
M+· m/z 389 (absent)
SO2C6F5
N
+
m/z 297.9 (100%)
SO2C6F5
+
m/z 230.9 (20.6%)
C6F5
+
m/z 166.9 (17.2%)
CH2
+
+
m/z 91 (35.0%)

Figure 2.7. Mass spectral fragmentation of N-propargylphenylethylamine derivatized with PFBSC. Percentages represent percent abundance relative to the base peak.

	PEA	N-methylPEA	N-propargylPEA
recovery (%, N=5)	92.7	102.7	83.1
intraassay reliability (%, N=5)	4.7	4.0	8.5
interassay reliability (%, N=5)	1.0	2.3	2.5

Table 2.1. Mean recovery and reliability results obtained with the assay procedure. Reliability values are expressed as coefficients of variance (C.V.s) calculated as (S.D./Mean) x 100%.

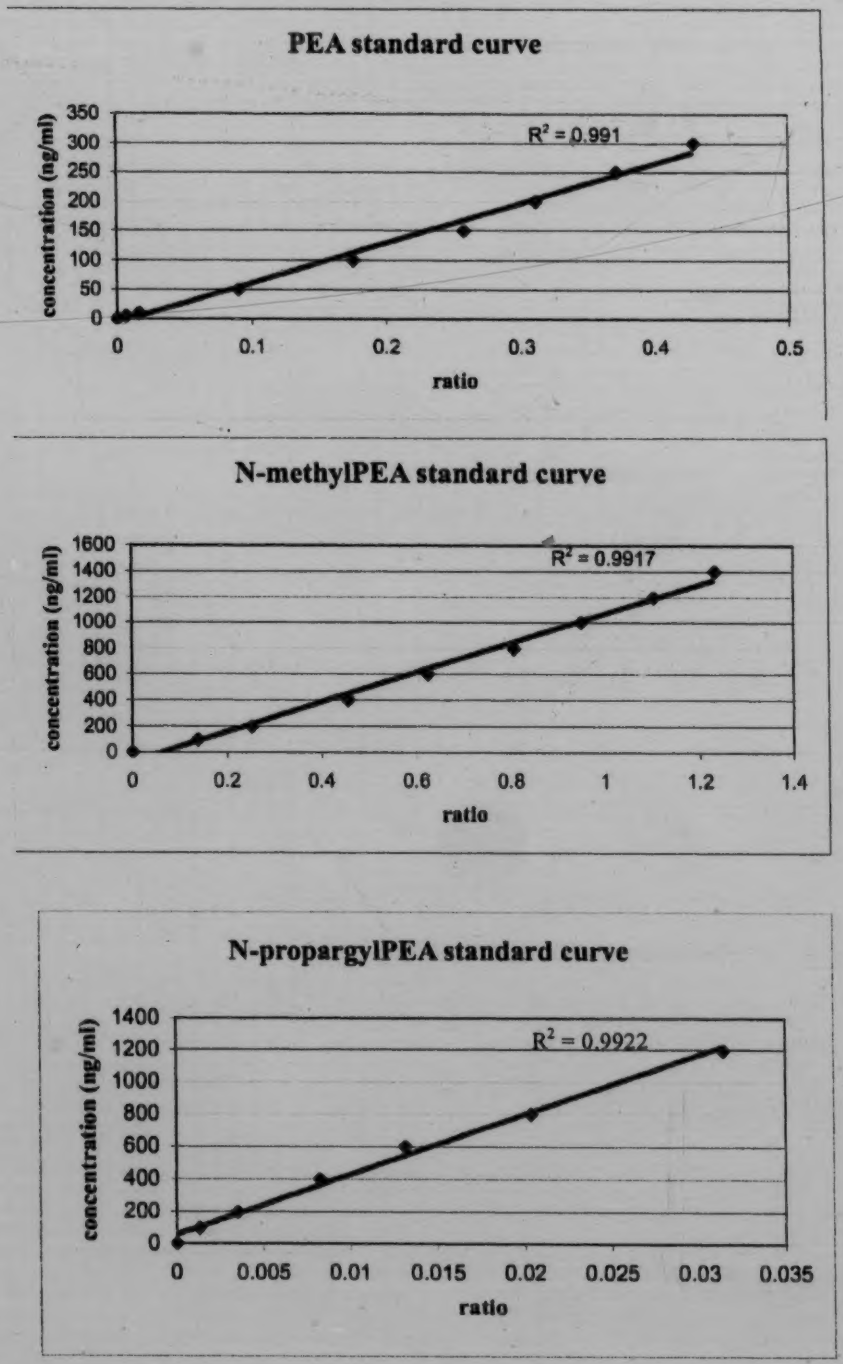


Figure 2.8: Typical standard curves for PEA, N-methylPEA and N-propargyPEA.

for N-methylPEA and N-propargylPEA; these values represent 10 pg and < 67 pg respectively "on column".

This procedure is currently being used in our laboratories to quantify metabolite formation from MPPE in the presence of HLMs. The results shown in Table 2.2 demonstrate increasing formation of all three metabolites with time.

2.4 DISCUSSION

Extractive derivatization with PFBSC under aqueous conditions followed by GC-ECD has been shown previously to be a rapid, sensitive method for the analysis of a wide variety of endogenous compounds and xenobiotics containing amine and/or phenol compounds (Sentissi et al., 1984; Baker et al., 1986a and 1986b; Nazarali *et al.*, 1987; Urichuk et al., 1997; Liu et al., 2002). Colleagues in our laboratories have used a similar method previously to study levels of amphetamine in rats or humans (Paetsch et al., 1992; Asghar et al., 2001) and to analyze levels of endogenous PEA in brain tissue from rats (Baker et al., 1986a). The method does not require preliminary procedures such as liquid-liquid extraction or solid phase extraction, as are often required in procedures involving derivatization under anhydrous conditions.

The procedure described in the current paper demonstrates that N-methylPEA and N-propargylPEA can be assayed simultaneously with PEA, which has not been previously shown using this method. The method has now been applied to human liver microsome studies to demonstrate that all three of these amines are metabolites of MPPE. Studies using the procedure are currently

Time (minutes)	PEA concentration (ng/ml)	N-methylPEA concentration (ng/ml)	N-propargylPEA concentration (ng/ml)
5	N/D	191.7	537.6
10	N/D	394.6	841.5
15	7.7	525.4	1251.8
20	15.1	649.4	1451.2
30	29.2	784.8	2235.1
60	65.8	1090.6	2320.2

N/D = not detectable

Table 2.2. Concentrations of metabolites of MPPE formed during incubation of MPPE (500 μM) with human liver microsomes at varying time intervals (N=3).

underway to investigate which cytochrome P450 enzymes catalyze these metabolic pathways. As indicated in the introduction to this chapter, the potential metabolites PEA, N-methylPEA and N-propargyPEA were investigated due to the known metabolism of deprenyl and the structural similarity of deprenyl and MPPE. The authors realize that other metabolites (eg. hydroxylated metabolites) may be formed in small quantities, as is the case for deprenyl (Shin, 1997; Taavitsainen et al., 2000; Tabi et al., 2003). Two small peaks in addition to those for PEA, N-methylPEA and N-propargylPEA are shown in the trace in Figure 2.4, but these were not consistently present in all samples studied.

2.5 REFERENCES

Am, O. B., Amit, T. and Youdim, M. B. H. (2004) Contrasting neuroprotective and neurotoxic actions of respective metabolites of anti-Parkinson drugs rasagiline and selegiline. *Neurosci Lett* 355:169-172

Asghar, S. J., Baker, G. B., Rauw, G. A. and Silverstone, P. H. (2001) A rapid method of determining amphetamine in plasma samples using pentafluorobenzenesulfonyl chloride and electron-capture gas chromatography. *J Pharmacol Toxicol* 46:111-5

Baker, G. B., Koilpillai, M., Nazarali, A. J., Rao, T. S. and Coutts, R. T. (1986a) Gas chromatography of antidepressants and their metabolites as pentafluorobenzoyl and pentafluorobenzenesulfonyl derivatives. *Proc West Pharmacol Soc* 29:291-294

Baker, G. B., Rao, T. S. and Coutts, R. T. (1986b) Electron-capture gas chromatographic analysis of beta-phenylethylamine in tissues and body fluids using pentafluorobenzenesulfonyl chloride for derivatization. *J Chromatogra Biomed Appl* 381:211-7

Birks, J. and Flicker, L. (2003) Selegiline for Alzheimer's disease. *Cochrane Database System Review* CD000442

DeLaGarza, V. W. (2003) Pharmacologic treatment of Alzheimer's disease: an update. *A Fam Phys* 68:1365-72

Ebadi, M., Sharma, S., Shavali, S. and El Refaey, H. (2002) Neuroprotective actions of selegiline. *J Neurosci Res* 67:285-9

Heinonen, E. H. and Myllyla, V. (1998) Safety of selegiline (deprenyl) in the treatment of Parkinson's disease. *Drug Safety* 19:11-22

Kwan, E., Baker, G. B., Shuaib, A., Ling, L. and Todd, K. G. (2000) N-Methyl,N-propargyl-2-phenylethylamine (MPPE), an analog of deprenyl, increases neuronal cell survival in thiamin deficiency encephalopathy. *Drug Devel Res* 51:244-252

Liu, Z. Q., Tan, Z. R., Wang, D., Huang, S. L., Wang, L. S. and Zhou, H. H. (2002) Simultaneous determination of fluoxetine and its metabolite p-trifluoromethylphenol in human liver microsomes using a gas chromatographic-electron-capture detection procedure. *J Chromatogr B Anal Tech Biomed Life Sci* 769:305-11

Magyar, K. and Szende, B. (2004) (-)-Deprenyl, a selective MAO-B inhibitor, with apoptotic and anti-apoptotic properties. *NeuroToxicol* 25:233-242

Mandel, S., Grunblatt, E., Riederer, P., Gerlach, M., Levites, Y. and Youdim, M. B. (2003) Neuroprotective strategies in Parkinson's disease : an update on progress. *CNS Drugs* 17:729-62

Nazarali, A. J., Baker, G. B., Coutts, R. T., Yeung, J. M. and Rao, T. S. (1987) Rapid analysis of beta-phenylethylamine in tissues and body fluids utilizing pentafluorobenzoylation followed by electron-capture gas chromatography. *Prog Neuropsychopharmacol Biol Psychiatry* 11:251-8

Paetsch, P. R., Baker, G. B., Caffaro, L. E., Greenshaw, A. J., Rauw, G. A. and Coutts, R. T. (1992) Electron-capture gas chromatographic procedure for simultaneous determination of amphetamine and N-methylamphetamine. *J Chromatogr Biomed Appl* 573:313-7

Paterson, I. A., Zhang, D., Warrington, R. C. and Boulton, A. A. (1998) R-deprenyl and R-2-heptyl-N-methylpropargylamine prevent apoptosis in cerebellar granule neurons induced by cytosine arabinoside but not low extracellular potassium. *J Neurochem* 70:515-23

Riederer, P. and Lachenmayer, L. (2003) Selegiline's neuroprotective capacity revisited. *J Neural Transm* 110:1273-8

Semkova, I., Wolz, P., Schilling, M. and Krieglstein, J. (1996) Selegiline enhances NGF synthesis and protects central nervous system neurons from excitotoxic and ischemic damage. *Eur J Pharmacol* 315:19-30

Sentissi, A., Joppich, M., O'Connell, K., Nazareth, A. and Giese, R. W. (1984) Pentafluorobenzenesulfonyl chloride: a new electrophoric derivatizing reagent with application to tyrosyl peptide determination by gas chromatography with electron capture detection. *Anal Chem* 56:2512-7

Shin, H. S. (1997) Metabolism of selegiline in humans. Identification, excretion, and stereochemistry of urine metabolites. *Drug Metab Dispos* 25:657-62

Sowa, B. N., Todd, K. G., Tanay, V. A. M. I., Holt, A. and Baker, G. B. (2004) Amine oxidase inhibitors and development of neuroprotective drugs. *Curr Neuropharmacol* 2:153-168

Stocchi, F. and Olanow, C. W. (2003) Neuroprotection in Parkinson's disease: clinical trials. *Ann Neurol* 53 Suppl 3:S87-97; discussion S97-9

Taavitsainen, P., Anttila, M., Nyman, L., Karnani, H., Salonen, J. S. and Pelkonen, O. (2000) Selegiline metabolism and cytochrome P450 enzymes: in vitro study in human liver microsomes. *Pharmacol Toxicol* 86:215-21

Tabi, T., Magyar, K. and Szoko, E. (2003) Chiral characterization of deprenyl-N-oxide and other deprenyl metabolites by capillary electrophoresis using a dual cyclodextrin system in rat urine. *Electrophoresis* 24:2665-73

Tatton, W. G. (1993) Selegiline can mediate neuronal rescue rather than neuronal protection. *Movement Disord* 8 Suppl 1:S20-30

Tatton, W. G. and Chalmers-Redman, R. M. (1996) Modulation of gene expression rather than monoamine oxidase inhibition: (-)-deprenyl-related compounds in controlling neurodegeneration. *Neurol* 47:S171-83

Tatton, W. G., Chalmers-Redman, R. M., Elstner, M., Leesch, W., Jagodzinski, F. B., Stupak, D. P., Sugrue, M. M. and Tatton, N. A. (2000) Glyceraldehyde-3-phosphate dehydrogenase in neurodegeneration and apoptosis signaling. *J Neural Transm Suppl* 77-100

Urichuk, L. J., Aspeslet, L. J., Holt, A., Silverstone, P. H., Coutts, R. T. and Baker, G. B. (1997) Determination of p-trifluoromethylphenol, a metabolite of fluoxetine, in tissues and body fluids using an electron-capture gas chromatographic procedure. *J Chromatogr B Biomed Sci Appl* 698:103-9

CHAPTER 3

METABOLISM OF N-METHYL,N-PROPARGYLPHENYLETHYLAMINE: STUDIES WITH HUMAN LIVER MICROSOMES AND cDNA EXPRESSED CYTOCHROME P450 (CYP) ENZYMES[2]

---

[2] A version of this chapter has been published. Rittenbach, K.A., Holt, A., Ling, L., Shan, J. and Baker, G.B. (2007) *Cellular and Molecular Neurobiology* 27: 179-190.

3.1 INTRODUCTION

Results from many *in vivo* and *in vitro* studies have shown that (-)-deprenyl (N-methyl,N-propargylamphetamine) has both neuroprotective and neurorescue properties (Tatton, 1993; Semkova et al., 1996; Tatton and Chalmers-Redman, 1996; Paterson et al., 1998; Todd and Butterworth, 1998; Tatton et al., 2000; Ebadi et al., 2002; Riederer and Lachenmayer, 2003; Magyar and Szende, 2004; Sowa et al., 2004). (-)-Deprenyl is a selective, irreversible monoamine oxidase (MAO)-B inhibitor, although at high concentrations, MAO-A is also inhibited. However, in many cases, the neuroprotective/neurorescue mechanisms involved seem to be independent of (-)-deprenyl's effects on MAO-B (Berry et al., 1994; Semkova et al., 1996). (-)-Deprenyl's major clinical use is in Parkinson's disease (PD), where it is proposed to delay development of motor symptoms and thus prolong the period before treatment with L-DOPA becomes necessary. Studies investigating efficacy in Parkinson's disease are extensive and generally show positive results, although some debate about whether these results represent true neuroprotection or simply indicate symptomatic relief is ongoing (Heinonen and Myllyla, 1998; Mandel et al., 2003; Stocchi and Olanow, 2003). (-)-Deprenyl may also be effective in delaying the progression of Alzheimer's disease (AD). Improvement in mental cognition, behaviour and mood in AD have been reported with (-)-deprenyl (DeLaGarza, 2003), but a large meta-analysis by Birks and Flicker (2003) concluded that there was no evidence of clinically meaningful benefit and so did not recommend its use.

That (-)-methamphetamine and (-)-amphetamine are major metabolites of (-)-deprenyl is a concern, as they are potential neurotoxins and may actually interfere with the neuroprotective action of deprenyl (Am et al., 2004). In contrast, the other major metabolite, N-propargylamphetamine, may be partially responsible for the neuroprotective efficacy of (-)-deprenyl (Mytilineou et al., 1997; Mytilineou et al., 1998; Maruyama and Naoi, 1999).

A novel analogue of deprenyl which is also a MAO inhibitor and which, like (-)-deprenyl, has been shown to be neuroprotective in the thiamine-deficiency model of neurotoxicity (Kwan et al., 2000) and in protecting against DSP-4 induced reduction in hippocampal noradrenaline (personal communication), is N-methyl,N-propargylphenylethylamine (MPPE). Metabolism of MPPE (Figure 3.1) would most likely parallel that of deprenyl, producing β-phenylethylamine (PEA), N-methylphenylethylamine (N-methylPEA) and N-propargylphenylethylamine (N-propargylPEA). That is, with MPPE, amphetamines would not be produced as metabolites. In order to investigate the metabolism of MPPE, a rapid, sensitive method of analysis for the three metabolites was developed in our laboratories. The procedure, which involves derivatization under aqueous conditions with pentafluorobenzenesulfonyl chloride (PFBSC) followed by analysis by GC-ECD, demonstrated in a preliminary study that PEA and its N-alkyl derivatives are indeed metabolites of MPPE (Rittenbach et al., 2005).

The availability of human liver microsomes (HLMs) and individual cDNA-expressed cytochrome P450 (CYP) enzymes has greatly facilitated studies of drug metabolism and drug-drug interactions (Salonen et al., 2003; Ring and

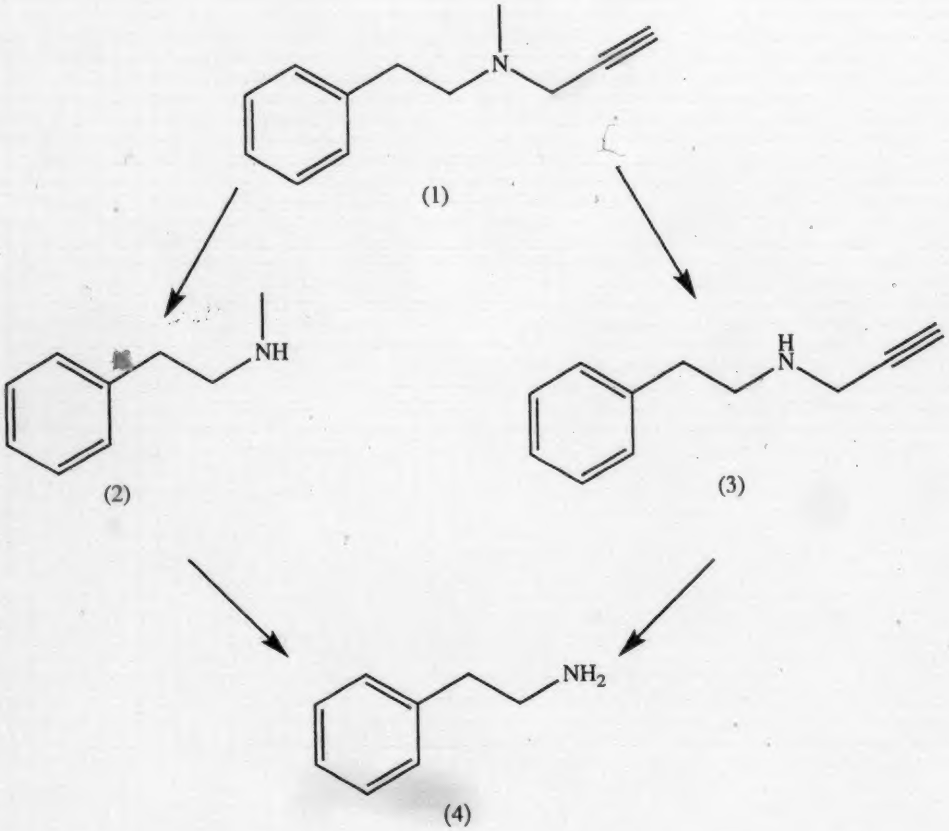


Figure 3.1. Proposed CYP-mediated metabolism of MPPE (1) to N-methylphenylethylamine (2), N-propargylphenylethylamine (3) and β-phenylethylamine (4).

Wrighton, 2000; Thummel et al., 2000). In this article, we present data from a fluorometric screening procedure which identifies interactions between MPPE and cDNA-expressed CYP enzymes, and a preliminary kinetic analysis of MPPE metabolism by human liver microsomes. Following initial screening, MPPE was incubated with cDNA-expressed CYPs 2B6, 2C19 and 2D6, and the contributions of these enzymes to the formation of N-methylPEA and N-propargylPEA were determined.

3.2 METHODS

3.2.1 Materials

β-Phenylethylamine (PEA) HCl, N-methylPEA, benzylamine HCl (internal standard, IS), glucose-6-phosphate (G6P), (-)-deprenyl, PFBSC and β-nicotinamide adenine dinucleotide phosphate (NADP, sodium salt) were purchased from the Sigma Chemical Company (St. Louis, MO, USA).

N-PropargylPEA HCl was synthesized for this study by Dr. E.E. Knaus in the Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta (Edmonton, AB, Canada). The structure was confirmed by nuclear magnetic resonance spectroscopy using a Bruker AM-300 spectrometer.

MPPE was supplied by CV Technologies Inc. (Edmonton, AB, Canada). Glucose-6-phosphate dehydrogenase (G6PD) was purchased from Cedarlane Laboratory Limited (Hornby, ON, Canada).

The HLM solution (HK-25), baculovirus-expressed CYP1A2, 2B6, 2C19, 2D6, 3A4, 3-cyano-7-ethoxycoumarin, 7-ethoxy-4-(trifluoromethyl)-coumarin, 3-

[2-(N,N-diethyl-N-methylammonium)ethyl]-7-hydroxy-4-methylcoumarin and 7-benzyloxy-4-(trifluoromethyl)-coumarin were purchased from Gentest Corporation (Woburn, MA, USA). HK-25 contained a total of 300 pmol P450 protein per mg of total protein and activity results were 25 pmol product per (mg protein x minute) for CYP2B6 using the (S)-mephenytoin N-demethylase assay, 200 pmol product per (mg protein x minute) for CYP2C19 using the (S)-mephenytoin 4'-hydroxylase, and 63 pmol product per (mg protein x minute) of bufuralol 1'-hydroxylase inhibited by 1 μM quinidine for CYP2D6.

Ethyl acetate, acetonitrile, toluene, potassium carbonate, potassium bicarbonate, monobasic potassium phosphate, dibasic potassium phosphate and magnesium chloride were purchased from Fisher Scientific (Mississauga, ON, Canada); all were HPLC or reagent grade and were used without further purification.

3.2.2 Incubations with human liver microsomes

An NADPH-generating solution was prepared containing 4 mg/ml (6.03 mM) NADP, 4 mg/ml (15.43 mM) G6P, 3.2 units/ml G6PD and 2.6 mg/ml (43.5 mM) magnesium chloride in potassium phosphate buffer (0.1 M, pH 7.4). The microsome incubation medium consisted of 25 μl of the NADPH-generating solution, 10 μl of microsomal solution, MPPE (10-500 μM) added in double-distilled water in a volume of between 5 μl and 25 μl and sufficient potassium phosphate buffer (0.1 M, pH 7.4) to bring the final volume to 100 μl. With each set of experimental parameters, two equimolar samples of (-)-deprenyl were included as a positive control. The metabolic reaction was allowed to proceed for 5-60 min in a 37°C water bath, after which the samples were placed on ice and 50 µl of 25% (1.8 M) potassium carbonate solution in water were added to stop the reaction. Double-distilled water (850 µl) and 40 µl of working IS solution (400 ng) were added and the samples were transferred to screw-top tubes for extraction. In a further experiment, 3.38 µl of N-methylPEA (1 mg/ml) or 4.0 µl of N-propargylPEA (1 mg/ml) were incubated with HLMs for 60 minutes and the resulting PEA measured using the same methods.

3.2.3 Sample Preparation, Derivatization and Analysis

The analysis of samples for N-methylPEA, N-propargylPEA and PEA has been described elsewhere (Rittenbach *et al.*, 2005). Briefly, the aqueous samples were basified and shaken with a solution of PFBSC in ethyl acetate/acetonitrile. The organic phase was retained and taken to dryness using a Savant Speed-vac evaporator. The residue was dissolved in toluene, and an aliquot of this solution was analyzed in a gas chromotagraph equipped with a capillary column and an electron-capture detector.

The samples for analyzed amphetamine and methamphetamine were prepared as above. The standard curves were linear and the R^2 value for amphetamine averaged 0.9897 (N=12) and for methamphetamine 0.9869 (N=14). N-Propargylamphetamine samples were basified with 250 µM of 25% potassium carbonate; 4 ml of ethyl acetate: PFBC (1 ml : 1 µl) were added and the tubes shaken for 10 minutes and centrifuged for 5 minutes. These samples were then treated the same as the other samples. The standard curves were linear and the R^2 value was 0.9881 (N=13).

3.2.4 Fluorogenic screen for interaction of MPPE with CYP enzymes

MPPE (200 nM - 20 mM) was preincubated with the NADPH-generating solution for 10 minutes at 37°C. The cDNA-expressed CYP enzyme and corresponding fluorogenic substrate were then added to the wells and incubated at 37°C (1A2 for 15 min., 2B6, 2C19, 2D6 and 3A4 for 30 min.). Enzyme activity was terminated by the addition of 75 μl of a 1:4 (v/v) solution of Tris (0.5 M) /acetonitrile. The fluorogenic substrates yield a fluorescent product upon metabolism by the appropriate CYP enzyme (Table 3.1). Product-derived fluorescence was measured in a SpectraMax Gemini EM plate reader. Velocity data obtained in the presence of MPPE at various concentrations were fitted with the non-linear regression facility of GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) to an equation for linear competitive inhibition (Equation 3.1).

$$v = \frac{Top + (Bottom - Top)}{1 + 10^{\log_{10}\left(K_i\left(1+\frac{[S]}{K_M}\right)\right) - \log_{10}[MPPE]}} \qquad \text{[Equation 3.1]}$$

In this equation, Top and Bottom refer to the horizontal upper and lower limits of the sigmoidal curve, respectively, and [S] and K_M are values relating to metabolism of the fluorogenic substrate. Fitting sigmoidal curves with this equation allows direct determination of a K_i value (inhibitor dissociation constant) for the inhibition of CYP activity by MPPE, and thus an estimate for K_M, should

Substrate	3-cyano-7-ethoxy-coumarin	7-ethyoxy-4-(trifluoromethyl)-coumarin	3-[2-(N,N-diethyl-N-methyl-ammonium) ethyl]-7-hydroxy-4-methylcoumarin	7-benzyloxy-4-(trifluoromethyl)-coumarin
CYP	1A2, 2C19	2B6	2D6	3A4
Product	3-cyano-7-hydroxy-coumarin	7-hydroxy-4-trifluoromethyl-coumarin	3-[2-(N,N-diethylamino)ethyl]-7-hydroxy-4-methylcoumarin hydrochoride	7-hydroxy-4-trifluoromethyl-coumarin
Product l_{ex}/l_{em}	$410_{ex}/460_{em}$ nm	409_{ex} /530_{em} nm	390_{ex} /460_{em} nm	409_{ex} /530_{em} nm

Table 3.1. Fluorogenic CYP substrates and excitation and emission wavelengths used in the present studies.

MPPE act as a substrate for the CYP enzyme of interest. In order to determine whether or not MPPE was indeed metabolized, further studies were completed.

3.2.5 Studies on metabolism of MPPE by c-DNA-expressed CYP enzymes

The microsome incubation medium consisted of 25 µl of an NADPH-generating solution, 10 µl of microsomal solution (cDNA expressed 2B6, 2C19 or 2D6), 8.8 µl of MPPE or 9.3 µl of deprenyl solution (to yield final concentrations of 500 µM) and sufficient buffer to bring the final volume to 100 µl. With each of the enzymes, an established substrate of the CYP enzyme under investigation was included as a positive control. These were, for 2B6, bupropion (metabolized to hydroxybupropion; Faucette et al., 2000; Hesse et al., 2000); for 2C19, mephenytoin (metabolized to hydroxymephenytoin; Salsali et al., 2000; Desta et al., 2002) and for 2D6, nortriptyline (metabolized to hydroxynortriptyline; Venkatakrishnan et al., 1999; Morita et al., 2000). Metabolic reactions were allowed to proceed for 120 min in a 37°C water bath, after which the samples were placed on ice and 50 µl of 25% potassium carbonate solution were added to stop the reaction. Double-distilled water (850 µl) and 40 µl of working IS solution (400 ng) were added and the samples were transferred to screw top tubes. Hydroxymephenytoin and hydroxynortriptyline were derivatized with PFBSC and analyzed by GC-ECD as described for the metabolites of MPPE. Hydroxybupropion was not derivatized, but was analyzed directly by HPLC using a modification of the procedure of Faucette et al. (2000). The column used was a

4.6 x 150 mm Waters Symmetry C_{18}, no internal standard was used and the run was isosyncratic at 85 % A (0.25% triethylamine and 0.1% formic acid in water) and 15% B (100% acetonitrile).

3.3 RESULTS

Data from fluorogenic studies with cDNA-expressed human enzymes indicated that MPPE interacted with CYP isozymes 2B6, 2C19 and 2D6 (Figure 3.2). No interactions with CYP1A2 or CYP3A4 were observed at the concentrations of MPPE used in the study. Dissociation constants (K_i values ± SEM) for the interaction of MPPE with CYP2B6, 2C19 and 2D6, determined by curve-fitting of data to Equation 1, were 43.4 ± 3.0 μM, 65.0 ± 8.8 μM and 53.9 ± 8.9 μM, respectively.

Further studies with individual CYP isozymes revealed that while CYP2B6, 2C19 and 2D6 all contributed to the generation of N-methylPEA, only CYP2B6 catalyzed N-demethylation to yield N-propargylPEA (Figure 3.3). Furthermore, CYP2B6-catalysed N-demethylation occurred more rapidly than did N-depropargylation. These experiments were run in parallel with positive controls for each CYP enzyme which demonstrated that the enzymes were viable and active.

MPPE (10, 30, 50, 100, 300 and 500 μM) was incubated with an individual donor human liver microsome panel, and the resulting PEA, N-methylPEA and N-propargylPEA concentrations were measured at 5, 10, 15, 20, 30 and 60 minutes. Measured concentrations of N-methylPEA and N-

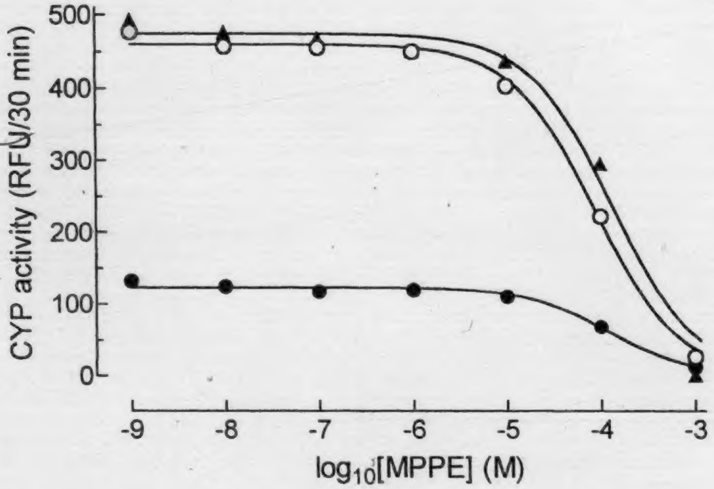


Figure 3.2. Inhibition by MPPE of metabolism of fluorogenic substrates by CYP2B6 (○), CYP2C19 (●) and CYP 2D6 (▲). Fluorogenic substrates were incubated with CYP isozymes at concentrations around their K_M values, in the presence of water (controls) or a range of concentrations of MPPE. Reaction velocities were expressed as a percentage of those in control samples. K_i values were obtained through fitting of data to Equation 1 for linear competitive inhibition (see text) (N=2). RFU = relative fluorescent units.

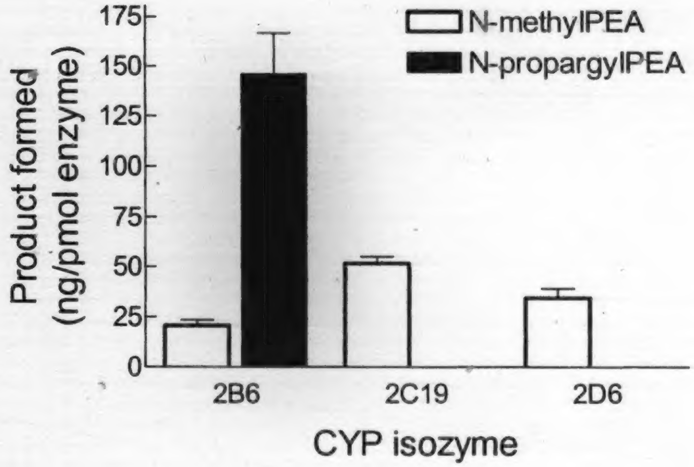


Figure 3.3. Rates of metabolism of MPPE (500 μM) by CYP isozymes identified as interacting with MPPE in fluorogenic screening assays. Each assay contained 10 μl of insect cell protein suspension containing CYP, as supplied by the manufacturer. Reaction velocities have been corrected for CYP content. Data represent mean ± SEM (N=4).

propargylPEA were plotted *versus* time (Figure 3.4) and the slope of the initial pseudolinear portion was taken as the initial velocity of formation (v) at each substrate concentration (Figure 3.5). With the nonlinear regression facility of GraphPad Prism, hyperbolic curve-fitting to the Michaelis-Menten equation resulted in K_M and V_{max} values of 290 ±70 μM and 139 ± 16 ng/ml/min for formation of N-propargylPEA, presumably by CYP2B6. However, formation of N-methylPEA is catalyzed by three enzymes, each with an associated K_M and V_{max} value, and curve-fitting to the Michaelis-Menten equation is not appropriate. Accordingly, data were fitted to Equation 3.2, for metabolism of a single substrate by three enzymes, with the nonlinear regression facility of GraphPad Prism.

$$v = \frac{V_{\max 1}[S]}{K_{M1} + [S]} + \frac{V_{\max 2}[S]}{K_{M2} + [S]} + \frac{V_{\max 3}[S]}{K_{M3} + [S]}$$

[Equation 3.2]

Nonlinear regression analysis with this equation did not permit independent determination of three pairs of K_M and V_{max} values, due to the relatively few data points available, to the number of variables in Equation 2 and to the consequent inherent variability in calculated kinetic constants. Thus, for illustrative purposes only, estimates for K_M and V_{max} values for the three contributing enzymes were made based on results presented in Figures 3.2 and 3.3, and on proportional CYP activities in the microsomes used, and these were held as constants in subsequent fitting of data for N-methylPEA to Equation 3.2 (Figure 3.5). PEA as a secondary metabolite was only measurable at higher initial

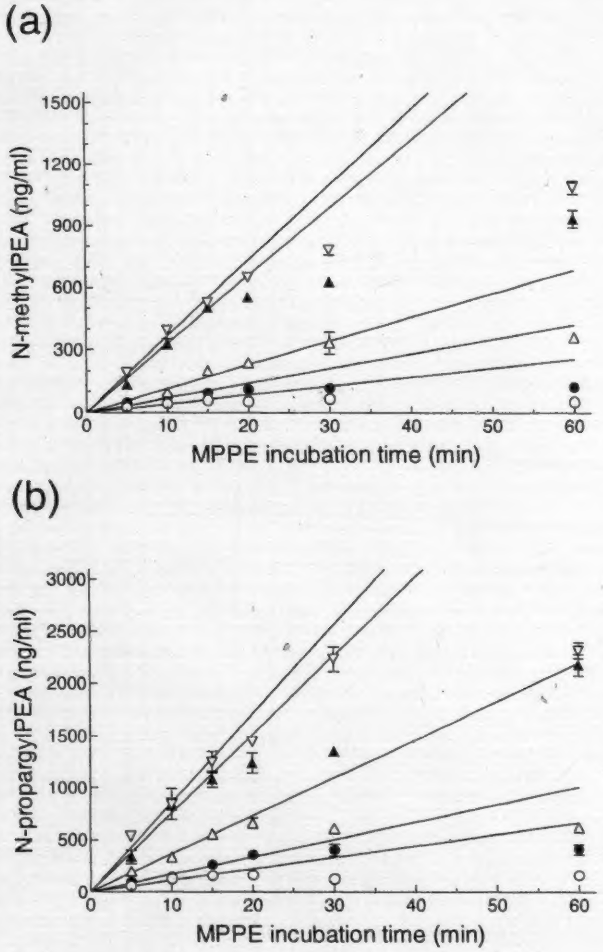


Figure 3.4. Time courses for CYP-mediated formation of N-methylPEA (a) and N-propargylPEA (b) from MPPE at 30 (○), 50 (●), 100 (△), 300 (▲) and 500 μM (▽) during incubation with HLMs from a single donor. Results represent means ± SEM (N=3); where error bars are smaller than the size of the symbol they are not shown. Each straight line represents the slope, and thus the initial velocity (v) during the pseudolinear (initial) portion of the time course, forced through the origin.

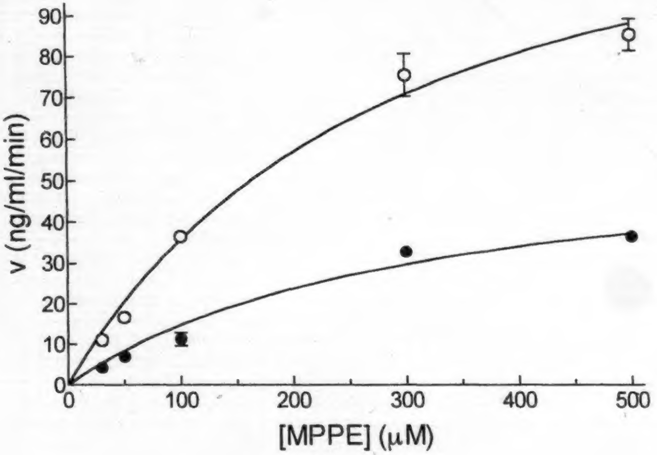


Figure 3.5. Kinetic plots for CYP-mediated generation of N-methylPEA (●) and N-propargylPEA (○) by HLMs. Initial velocities (v) were obtained from Figure 3.4. Data represent slopes ± SEM obtained by a single linear regression of mean values from triplicate determinations (see Figure 3.4). Generation of N-propargylPEA was by a single enzyme (CYP2B6; Figure 3.3); K_M and V_{max} values were determined by fitting data to the Michaelis-Menten equation (see text). Formation of N-methylPEA was catalyzed by three enzymes; data were thus fitted to an equation for three enzyme activities metabolising a single substrate (Equation 3.2). Nonlinear regression generated unacceptable values for constants, although a reasonable fit could be obtained by constraining constants at estimated values based on results shown in Figures 3.2 and 3.3, and estimated isozyme content of microsomes provided by the manufacturer (see Discussion). Respective paired V_{max} and K_M values for CYP isozymes were thus constrained to 26 ng/ml/min and 260 μM (CYP2B6), 175 ng/ml/min and 390 μM (CYP2C19) and 18 ng/ml/min and 323 μM (CYP2D6).

MPPE concentrations or longer incubation times, and data obtained for this metabolite are not shown. When N-methylPEA and N-propargylPEA were incubated with HLMs at 250 μM, only N-propargylPEA was metabolized to PEA (Figure 3.6).

(-)-Deprenyl was included in the study at 500 μM as a positive control, and indeed substantial amounts of N-methylamphetamine and N-propargylamphetamine were formed from (-)-deprenyl, as has been reported elsewhere in studies with human liver microsomes (Grace et al., 1994; Taavitsainen et al., 2000; Hidestrand et al., 2001; Salonen et al., 2003). A comprehensive comparison of the metabolism of the two drugs by cDNA-expressed microsomes under identical incubation conditions will be described elsewhere.

3.4 DISCUSSION

Metabolism of drugs to active metabolites or drug-drug interactions at the level of metabolism are of major clinical relevance (Harvey and Preskorn, 1996; Nemeroff et al., 1996; Kennedy et al., 2001), especially in the field of neuropsychiatry, where many patients are subjected to polypharmaceutical regimens. As indicated in the introduction to this paper, MPPE has shown neuroprotective effects similar to those of (-)-deprenyl in two animal models of neurotoxicity (Kwan et al., 2000; personal communication). The present study confirms the hypothesis that MPPE is metabolized to PEA, N-methylPEA and N-

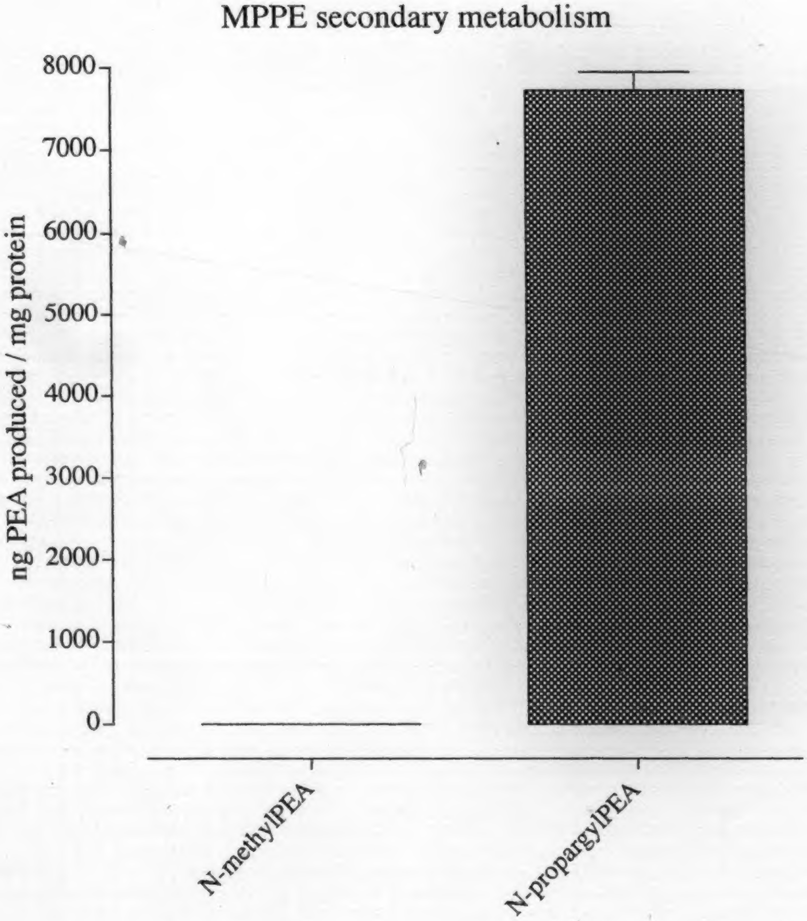


Figure 3.6. Amount of PEA produced / mg protein when 250 μM N-methylPEA and N-propargylPEA were incubated for 60 min. with HLMs (N = 4).

propargylPEA by human liver microsomes and reports the rate of formation of the two major metabolites of the drug, N-methylPEA and N-propargylPEA, by a human microsome preparation. Since N-propargylamphetamine has been proposed to contribute to the neuroprotective efficacy of (-)-deprenyl, it would be of interest in the future to determine if N-propargylPEA, the corresponding metabolite of MPPE, also has neuroprotective properties.

Data from a fluorescence-based study examining inhibition of cDNA-expressed CYP isozymes indicated that MPPE interacts with CYP2B6, 2C19 and 2D6, although such studies do not indicate if MPPE is acting as both a substrate and inhibitor of these enzymes. Further chromatographic studies involving incubation of MPPE with the individual cDNA-expressed CYP enzymes showed that metabolism of MPPE is catalyzed by all three of these isozymes (Rittenbach and Baker, 2007). However, while formation of N-methylPEA is catalyzed by all of the enzymes, only CYP2B6 contributes to formation of N-propargylPEA. The rate of N-propargylPEA formation from MPPE (500 μM) in the presence of CYP2B6 was approximately 7-fold higher than that for N-methylPEA formation by the same enzyme. At equimolar enzyme concentrations, CYP2C19 was most active with respect to generation of N-methylPEA, exhibiting a rate 50% higher than that with CYP2D6, and 150% higher than that with CYP2B6 (Figure 3.3). Although the concentration of MPPE (500 μM) was identical in assays with the three different isozymes, velocities are directly comparable given the similarities in K_i values determined in earlier fluorogenic screening assays.

It was found that a three-enzyme curve could not be fitted successfully to kinetic data for generation of N-methylPEA (Figure 3.5) unless K_M values were held at values several (5-10) fold higher than those predicted from K_i determinations. Further CYP-mediated N-dealkylation of one of the primary metabolites of MPPE yields PEA, the presence of which has been confirmed in these and other studies (Rittenbach et al., 2005). Fitting of data to Equation 3.2 does not take into account the likelihood that one or both N-dealkylated metabolites of MPPE interact as substrate(s) with the active site of one or more of the CYP enzymes of interest, the result of which would be competitive inhibition of MPPE turnover and an increase in the apparent K_M value for metabolism of MPPE, relative to the measured K_i. The substantial increase in K_M values, compared with respective K_i values, observed here indicates that the affinities of one or both N-dealkylated metabolites for CYP isozymes may be rather higher than that of the parent drug, MPPE. Further studies with these primary metabolites are warranted.

Studies with N-methylPEA and N-propargylPEA demonstrated that N-propargylPEA is metabolized by HLMs to PEA, while surprisingly, N-methylPEA incubations did not result in measurable levels of PEA. This could be due to PEA resulting from N-methylPEA incubations being metabolized further, for example by hydroxylation. However, this argument would also presumably apply to PEA formed from N-propargylPEA unless it was formed at a different rate. It is interesting that N-methylPEA levels decreased during the incubation with HLMs, suggesting that it was metabolized by the HLMs, perhaps through direct hydroxylation of N-methylPEA, perhaps bypassing PEA formation.

3.5 REFERENCES

Am, O. B., Amit, T. and Youdim, M. B. H. (2004). Contrasting neuroprotective and neurotoxic actions of respective metabolites of anti-Parkinson drugs rasagiline and selegiline. *Neurosci Lett*. 355:169-172

Berry, M. D., Juorio, A. V. and Paterson, I. A. (1994). Possible mechanisms of action of (-)-deprenyl and other MAO-B inhibitors in some neurologic and psychiatric disorders. *Prog Neurobiol*. 44:141-161

Birks, J. and Flicker, L. (2003). Selegiline for Alzheimer's disease. *Cochrane Database System Review*. CD000442

DeLaGarza, V. W. (2003). Pharmacologic treatment of Alzheimer's disease: an update. *Am Fam Physician*. 68:1365-1372

Desta, Z., Zhao, X., Shin, J. G. and Flockhart, D. A. (2002). Clinical significance of the cytochrome P450 2C19 genetic polymorphism. *Clin Pharmacokinet*. 41:913-958

Ebadi, M., Sharma, S., Shavali, S. and El Refaey, H. (2002). Neuroprotective actions of selegiline. *J Neurosci Res*. 67:285-289

Faucette, S. R., Hawke, R. L., Lecluyse, E. L., Shord, S. S., Yan, B., Laethem, R. M. and Lindley, C. M. (2000). Validation of bupropion hydroxylation as a selective marker of human cytochrome P450 2B6 catalytic activity. *Drug Metab Dispos*. 28:1222-1230

Grace, J. M., Kinter, M. T. and Macdonald, T. L. (1994). Atypical metabolism of deprenyl and its enantiomer, (S)-(+)-N,alpha-dimethyl-N-propynylphenethylamine, by cytochrome P450 2D6. *Chem Res Toxicol*. 7:286-290

Harvey, A. T. and Preskorn, S. H. (1996). Cytochrome P450 enzymes: Interpretation of their interactions with selective serotonin reuptake inhibitors Part 1. *J Clin Psychopharmacol*. 16:273-285

Heinonen, E. H. and Myllyla, V. (1998). Safety of selegiline (deprenyl) in the treatment of Parkinson's disease. *Drug Safety*. 19:11-22

Hesse, L. M., Venkatakrishnan, K., Court, M. H., von Moltke, L. L., Duan, S. X., Shader, R. I. and Greenblatt, D. J. (2000). CYP2B6 mediates the in vitro hydroxylation of bupropion: potential drug interactions with other antidepressants. *Drug Metab Dispos.* 28:1176-1183

Hidestrand, M., Oscarson, M., Salonen, J. S., Nyman, L., Pelkonen, O., Turpeinen, M. and Ingelman-Sundberg, M. (2001). CYP2B6 and CYP2C19 as the major enzymes responsible for the metabolism of selegiline, a drug used in the treatment of Parkinson's disease, as revealed from experiments with recombinant enzymes. *Drug Metab Dispos*. 29:1480-1484

Kennedy, S. H., Lam, R. W., Cohen, N. L., Ravindran, A. V. and Group, t. C. D. W. (2001). Clinical guidelines for the treatment of depression disorders IV. Medications and other biological treatments. *Can J Psychiatry*. 46:38S-58S

Kwan, E., Baker, G. B., Shuaib, A., Ling, L. and Todd, K. G. (2000). N-Methyl,N-propargyl-2-phenylethylamine (MPPE), an analog of deprenyl, increases neuronal cell survival in thiamin deficiency encephalopathy. *Drug Develop Res*. 51:244-252

Magyar, K. and Szende, B. (2004). (-)-Deprenyl, a selective MAO-B inhibitor, with apoptotic and anti-apoptotic properties. *NeuroToxicol*. 25:233-242

Mandel, S., Grunblatt, E., Riederer, P., Gerlach, M., Levites, Y. and Youdim, M. B. (2003). Neuroprotective strategies in Parkinson's disease : an update on progress. *CNS Drugs*. 17:729-762

Maruyama, W. and Naoi, M. (1999). Neuroprotection by (-)-deprenyl and related compounds. *Mech Ageing Dev*. 111:189-200

Morita, S., Shimoda, K., Someya, T., Yoshimura, Y., Kamijima, K. and Kato, N. (2000). Steady-state plasma levels of nortriptyline and its hydroxylated metabolites in Japanese patients: impact of CYP2D6 genotype on the hydroxylation of nortriptyline. *J Clin Psychopharmacol*. 20:141-149

Mytilineou, C., Leonardi, E. K., Radcliffe, P., Heinonen, E. H., Han, S. K., Werner, P., Cohen, G. and Olanow, C. W. (1998). Deprenyl and desmethylselegiline protect mesencephalic neurons from toxicity induced by glutathione depletion. *J Pharmacol Exp Ther*. 284:700-706

Mytilineou, C., Radcliffe, P. M. and Olanow, C. W. (1997). L-(-)-desmethylselegiline, a metabolite of selegiline [L-(-)-deprenyl], protects mesencephalic dopamine neurons from excitotoxicity in vitro. *J Neurochem*. 68:434-436

Nemeroff, C. B., DeVane, C. L. and Pollock, B. G. (1996). Newer antidepressants and the Cytochrome P450 system. *Am J Psychiatry*. 153:311-320

Paterson, I. A., Zhang, D., Warrington, R. C. and Boulton, A. A. (1998). R-deprenyl and R-2-heptyl-N-methylpropargylamine prevent apoptosis in cerebellar granule neurons induced by cytosine arabinoside but not low extracellular potassium. *J Neurochem*. 70:515-523

Riederer, P. and Lachenmayer, L. (2003). Selegiline's neuroprotective capacity revisited. *J Neural Transm*. 110:1273-1278

Ring, B. J. and Wrighton, S. A. (2000). Industrial viewpoint: application on *in vitro* drug metabolism in various phases of drug development. In R. H. Levy, K. E. Thummel, W. F. Trager, P. D. Hansten and M. Eichelbaum (ed.), *Metabolic Drug Interactions,* Lippincott Williams and Wilkins, Philadelphia, pp. 29-40

Rittenbach, K. and Baker, G. B. (2007). Involvement of cytochrome P450 2D6, 2B6 and 2C19 in the metabolism of (-)-deprenyl and N-methyl,N-propargylphenylethylamine. *Drug Metab Lett*. 1: 97-100.

Rittenbach, K., Sloley, B. D., Ling, L., Coutts, R. T., Shan, J. and Baker, G. B. (2005). A rapid, sensitive electron-capture gas chromatographic procedure for analysis of metabolites of N-methyl,N-propargylphenylethylamine, a potential neuroprotective agent. *J Pharmacol Toxicol Methods*. 52:373-378

Salonen, J. S., Nyman, L., Boobis, A. R., Edwards, R. J., Watts, P., Lake, B. G., Price, R. J., Renwick, A. B., Gomez-Lechon, M.-J., Castell, J. V., Ingelman-Sundberg, M., Hidestrand, M., Guillouzo, A., Corcos, L., Goldfarb, P. S., Lewis, D. F. V., Taavitsainen, P. and Pelkonen, O. (2003). Comparative studies on the cytochrome P450-associated metabolism and interaction potential of selegiline between human liver-derived in vitro systems. *Drug Metab Dispos*. 31:1093-1102

Salsali, M., Coutts, R. T. and Baker, G. B. (2000). A rapid electron-capture gas chromatographic procedure for the analysis of p-hydroxymephenytoin. *J Pharmacol Toxicol Methods*. 44:461-465

Semkova, I., Wolz, P., Schilling, M. and Krieglstein, J. (1996). Selegiline enhances NGF synthesis and protects central nervous system neurons from excitotoxic and ischemic damage. *Eur J Pharmacol*. 315:19-3

Sowa, B. N., Todd, K. G., Tanay, V. A. M. I., Holt, A. and Baker, G. B. (2004). Amine oxidase inhibitors and development of neuroprotective drugs. *Curr Neuropharmacol*. 2:153-168

Stocchi, F. and Olanow, C. W. (2003). Neuroprotection in Parkinson's disease: clinical trials. *Ann Neurol*. 53 Suppl 3:S87-97; discussion S97-89

Taavitsainen, P., Anttila, M., Nyman, L., Karnani, H., Salonen, J. S. and Pelkonen, O. (2000). Selegiline metabolism and cytochrome P450 enzymes: in vitro study in human liver microsomes. *Pharmacol Toxicol.* 86:215-221

Tatton, W. G. (1993). Selegiline can mediate neuronal rescue rather than neuronal protection. *Movement Disorders*. 8 Suppl 1:S20-30

Tatton, W. G. and Chalmers-Redman, R. M. (1996). Modulation of gene expression rather than monoamine oxidase inhibition: (-)-deprenyl-related compounds in controlling neurodegeneration. *Neurol.* 47:S171-18

Tatton, W. G., Chalmers-Redman, R. M., Elstner, M., Leesch, W., Jagodzinski, F. B., Stupak, D. P., Sugrue, M. M. and Tatton, N. A. (2000). Glyceraldehyde-3-phosphate dehydrogenase in neurodegeneration and apoptosis signaling. *J Neural Transm Supplement.* 77-100

Thummel, K. E., Kunze, K. L. and Shen, D. D. (2000). Metabolically-based drug-drug interactions: principles and mechanisms. In R. H. Levy, K. E. Thummel, W. F. Trager, P. D. Hansten and M. Eichelbaum (eds.), *Metabolic Drug Interactions,* Lippincott Williams and Wilkins, Philadelphia, pp. 3-21

Todd, K. G. and Butterworth, R. F. (1998). Increased neuronal cell survival after L-deprenyl treatment in experimental thiamine deficiency. *J Neurosci Res.* 52:240-246

Venkatakrishnan, K., von Moltke, L. L. and Greenblatt, D. J. (1999). Nortriptyline E-10-hydroxylation in vitro is mediated by human CYP2D6 (high affinity) and CYP3A4 (low affinity): implications for interactions with enzyme-inducing drugs. *J Clin Pharmacol.* 39:567-577

CHAPTER 4

INVOLVEMENT OF CYTOCHROMES P450 2D6, 2B6 AND 2C19 IN THE METABOLISM OF (-)-DEPRENYL AND N-METHYL,N-PROPARGYLPHENYLETHYLAMINE.[3]

---

[3] A version of this chapter has been published. Rittenbach, K.A. and Baker, G.B. (2007) *Drug Metabolism Letters* 1: 97-100.

4.1 INTRODUCTION

The current clinical use for the irreversible monoamine oxidase (MAO)-B inhibitor N-methyl,N-propargylamphetamine [(-)-deprenyl] is in Parkinson's disease, where it can delay the onset of motor symptoms and delay the need for L-dopa therapy (Thomas, 2000, Mandel, *et al.*, 2003, Riederer and Lachenmayer, 2003, Olanow, 2004, Schapira and Olanow, 2004). However, it is also being investigated for use in Alzheimer's disease (DeLaGarza, 2003), attention deficit disorder (Akhondzadeh, *et al.*, 2003), cocaine addiction [(McCann, *et al.*, 1999), but see also (Elkashef, *et al.*, 2006)], and transdermally as an antidepressant (Patkar, *et al.*, 2006). In numerous in vitro and in vivo experiments (-)-deprenyl has demonstrated neuroprotective and/or neurorescue effects (Tatton, 1993, Semkova, *et al.*, 1996, Tatton and Chalmers-Redman, 1996, Paterson, *et al.*, 1998, Todd and Butterworth, 1998, Stocchi and Olanow, 2003, for review: Sowa, *et al.*, 2004).

Although the main metabolites of (-)-deprenyl have been identified and measured, the enzymes involved in the formation of these metabolites have been the subject of debate for several years. In humans, the main metabolites are amphetamine, methamphetamine and N-propargylamphetamine (Shin, 1997) (see Figure 4.1) and minor metabolites include hydroxylated compounds and the more recently identified deprenyl-N-oxide (Tabi, *et al.*, 2003).

In 1994, Grace et al. reported that (-)-deprenyl was an "atypical" substrate of cytochrome P450 (CYP)2D6 (Grace, *et al.*, 1994). This study used cDNA-expressed CYP2D6 from yeast cells and reported the formation of both major

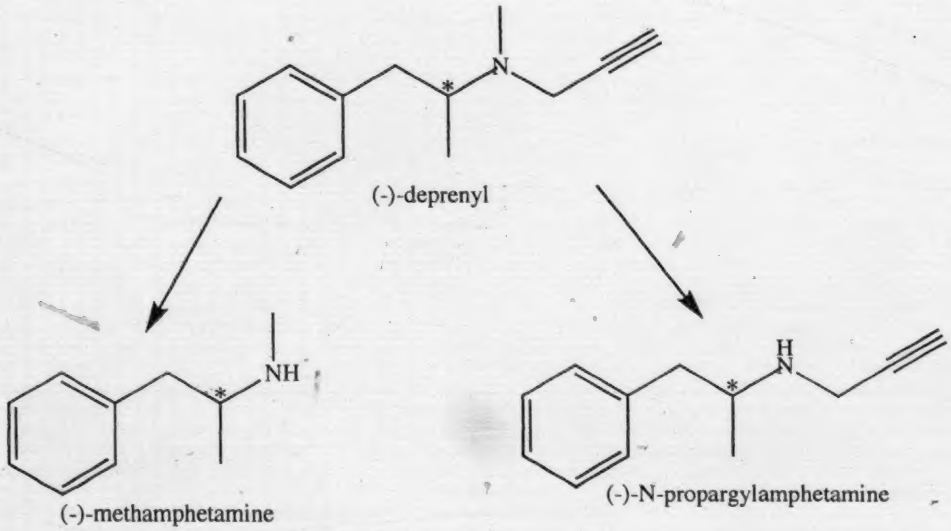

(-)-deprenyl
(-)-methamphetamine
(-)-N-propargylamphetamine

Figure 4.1. Metabolism of (-)-deprenyl to methamphetamine and N-propargylamphetamine. Both methamphetamine and N-propargylamphetamine can further be metabolized to amphetamine.

metabolites of (-)-deprenyl, methamphetamine and N-propargylamphetamine. Other reports supported the involvement of CYP2D6 (Sharma, *et al.*, 1996) and also 3A involvement (Wacher, *et al.*, 1996). In 1998, Scheinin et al. reported that CYP2D6 polymorphism did not affect (-)-deprenyl metabolism, implying that CYP2D6 was not of primary importance in the elimination of (-)-deprenyl (Scheinin, *et al.*, 1998).

In 2000 a study utilizing HLM preparations (Taavitsainen, *et al.*, 2000) showed involvement of CYPs 1A2, 3A4 and 2C19, but not 2D6, in metabolism of (-)-deprenyl. Using known inhibitors of CYP these authors looked at the involvement of individual CYP enzymes in formation of metabolites and reported that CYP1A2 catalyzed depropargylation but not demethylation and that CYP3A4 catalyzed both pathways (Taavitsainen, *et al.*, 2000). Another study published that year (Bach, *et al.*, 2000) indicated CYP2D6 involvement once again, using a fortified expressed CYP2D6 preparation.

Hidestrand et al. (2001) reported that CYP2B6 and CYP2C19 were the major enzymes responsible for the metabolism of (-)-deprenyl. This study used human cDNA expressed in yeast cells to investigate the involvment of CYPs 1A1, 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1 and 3A4 in the metabolism. The results showed that approximately 80% of (-)-deprenyl clearance was mediated by CYP2B6 and 2C19, and that 1A2 and 3A4 contributed approximately 15% more (Hidestrand, *et al.*, 2001).

These contradictory results may be due to the different experimental systems used and natural compensation by alternative pathways present in HLM preparations and in human subjects such as 2D6 poor metabolizers.

N-Methyl,N-propargylphenylethylamine (MPPE) is a structural analogue of (-)-deprenyl that differs from deprenyl only in the absence of the methyl group in the alpha position of the side chain. Like (-)-deprenyl, MPPE is neuroprotective in the thiamine deficiency model (Kwan, *et al.*, 2000). We have shown that the metabolism of MPPE parallels that of (-)-deprenyl, resulting in formation of N-methylphenylethylamine (N-methylPEA), N-propargylphenylethylamine (N-propargylPEA) and PEA instead of potentially damaging amphetamines (Rittenbach, K., *et al.*, 2005) (see Figure 4.2). We have also, in a screen with fluorogenic substrates, elucidated the CYP enzymes with the strongest interactions with MPPE, i.e. CYPs 2B6, 2C19 and 2D6, and completed kinetic studies of metabolite formation using those cDNA expressed enzymes (Rittenbach, K.A., *et al.*, 2007). The present paper reports on the comparison of (-)-deprenyl and MPPE metabolism by cDNA-expressed CYPs 2B6, 2C19 and 2D6 in experiments conducted under the same conditions, i.e. equimolar amounts of (-)-deprenyl and MPPE with the same amounts of enzyme for the same incubation time.

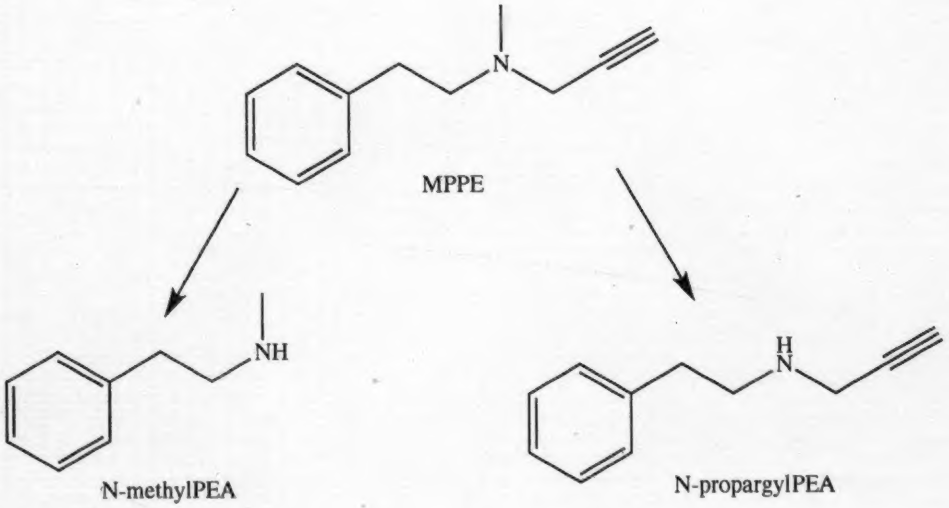

MPPE
NH
N-methylPEA
H
N
N-propargylPEA
N

Figure 4.2. Metabolism of MPPE to N-methylPEA and N-propargylPEA. Both N-methylPEA and N-propargylPEA can be further metabolized to PEA.

4.2 METHODS

4.2.1 Materials

β-Phenylethylamine (PEA) HCl, N-methylPEA, benzylamine HCl (internal standard, IS), glucose-6-phosphate (G6P), (-)-deprenyl, pentafluorobenzenesulfonyl chloride (PFBSC) and β-nicotine adenine dinucleotide phosphate (NADP) were purchased from Sigma Chemical Company (St. Louis, MO, USA). N-PropargylPEA HCl was synthesized by Dr. E.E. Knaus in the Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta (Edmonton, AB, Canada). MPPE was synthesized in the laboratories of CV Technologies (Edmonton, AB, Canada). Glucose-6-phosphate dehydrogenase (G6PD) was purchased from Cederlane Laboratory Limited (Hornby, ON, Canada). Baculovirus-expressed human CYPs 2B6, 2C19, and 2D6 were purchased from Gentest Corporation (Woburn, MA, USA).

Ethyl acetate, acetonitrile, toluene, potassium carbonate, potassium bicarbonate, potassium phosphate monobasic, potassium phosphate dibasic and magnesium chloride were purchased from Fisher Scientific (Mississauga, ON, Canada); all were HPLC or reagent grade and were used without further purification.

4.2.2 Studies on metabolism of MPPE using cDNA-expressed CYP enzymes

The microsome incubation medium consisted of 25 μl of an NADPH-generating system (4 mg/ml β-nicotine adenine dinucleotide phosphate, 4 mg/ml glucose-6-phosphate, 3.2 units/ml of glucose-6-phosphate dehydrogenase and 2.6

mg/ml of magnesium chloride in 0.1M potassium phosphate buffer (pH=7.4)), 10 µl of microsomal solution (cDNA-expressed 2B6, 2C19 or 2D6), 8.8 µl of MPPE solution (1mg/ml or 1 mg/10 ml) or 9.3 µl of (-)-deprenyl solution (1 mg/ml or 1 mg/10 ml) (resulting in final concentrations of 500 µM and 50 µM respectively) and sufficient buffer to result in a volume of 100 µl. The CYP content of the cDNA-expressed microsomes was 120, 30 and 40 pmol/mg for 2B6, 2C19 and 2D6 respectively. With each of the enzymes a known substrate of the CYP enzyme under investigation was included as a positive control. For 2B6 the positive control was buproprion (metabolized to hydroxybuproprion) (Faucette, *et al.*, 2000, Hesse, *et al.*, 2000), for 2C19 the positive control was mephenytoin (metabolized to hydroxymephenytoin) (Salsali, *et al.*, 2000, Desta, *et al.*, 2002) and for 2D6 nortriptyline metabolized to hydroxynortriptyline was used (Venkatakrishnan, *et al.*, 1999, Morita, *et al.*, 2000). The metabolic reaction was allowed to proceed for 120 min in a 37°C water bath, after which the samples were placed on ice and 50 µl of 25% potassium carbonate solution or acetonitrile (in the case of buproprion) were added to stop the reaction.

Double-distilled water (850 µl) and 40 µl of working IS solution (400 ng) were added and the samples were transferred to screw top tubes, except for the buproprion samples. Hydroxymephenytoin and hydroxynortriptyline were derivatized with PFBSC and analyzed by gas chromatography with electron-capture detection (GC-ECD) as described for the metabolites of MPPE (Rittenbach, *et al.*, 2005). Hydroxybuproprion was not derivatized, but was analyzed directly by HPLC using a modification of the procedure of Faucette et al. (Faucette, *et al.*, 2000). The column used was a 4.6 x 150 mm Waters Symmetry C18, no internal standard was used and the run was isosyncratic at 85 % A (0.25% triethylamine and 0.1% formic acid in water) and 15% B (100% acetonitrile).

4.2.3 Sample Preparation, Derivatization and Analysis

The analysis of samples for metabolites of MPPE and (-)-deprenyl has been described elsewhere (Rittenbach, *et al.*, 2005). Briefly, the aqueous samples are basified and shaken with a solution of PFBSC in ethyl acetate/acetonitrile, except for (-)-deprenyl samples for N-propargylamphetamine analysis, which are basified and shaken with a solution of PFBC in ethyl acetate. The organic phase is retained and taken to dryness. The residue is taken up in toluene, and an aliquot of this solution is analyzed using a gas chromatograph equipped with a capillary column and an electron-capture detector.

4.2.4 Statistics

All interactions were analyzed by two way ANOVA calculated with GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) followed, where necessary, by the Bonferroni post tests.

4.3 RESULTS

The results from incubating MPPE and (-)-deprenyl at 500 μM for 120 minutes at 37°C are summarized in Figure 4.3 and the results from 50 μM in

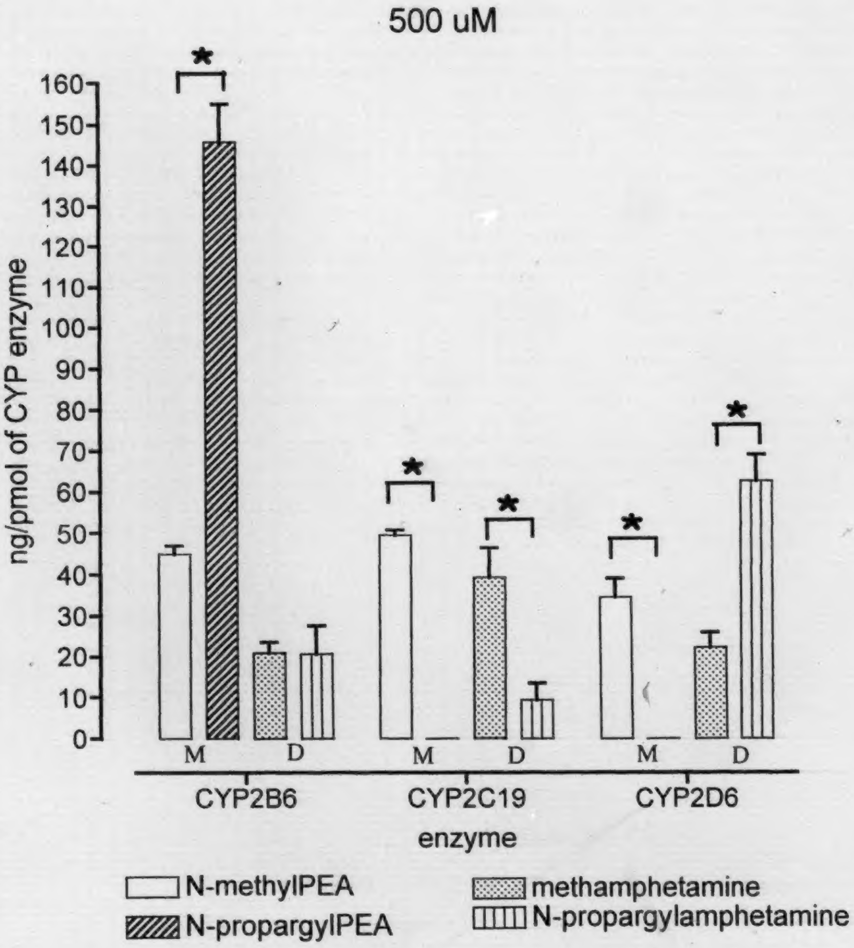


Figure 4.3. Results of incubation of MPPE (M) and (-)-deprenyl (D) (500 μM each) with cDNA-expressed CYP enzymes for 120 minutes. Results are expressed as means ± SEM (N=4). * = $p < 0.05$.

Figure 4.4. All reactions demonstrated a significant dose response when 50 μM and 500 μM were compared except the formation of methamphetamine and N-propargylamphetamine from (-)-deprenyl by CYP2C19.

CYP2D6 catalyzed the metabolism of both compounds, but some interesting differences were observed. At both concentrations CYP2D6 catalyzed the N-depropargylation, but not the N-demethylation, of MPPE and catalyzed both the N-depropargylation and N-demethylation of (-)-deprenyl. CYP2C19 catalyzed the N-depropargylation of both drugs at both concentrations, had no effect on N-demethylation of MPPE at either concentration, and catalyzed the N-demethylation of (-)-deprenyl in a variable way. CYP2B6 catalyzed the depropargylation and demethylation of both MPPE and (-)-deprenyl, but the amount of N-propargylPEA formed from MPPE was significantly greater than the amount of N-propargylamphetamine formed from (-)-deprenyl at both concentrations. In contrast, the difference in the degree of N-depropargylation (i.e. formation of N-methylPEA and methamphetamine) was much less at 500 μM than at 50 μM.

4.4 DISCUSSION

The differences between the metabolic profiles of these two drugs are of interest in part to help elucidate (-)-deprenyl metabolic pathways but more so because the chemical structures are very similar and the differences would appear to be the result of a single change at the α carbon.

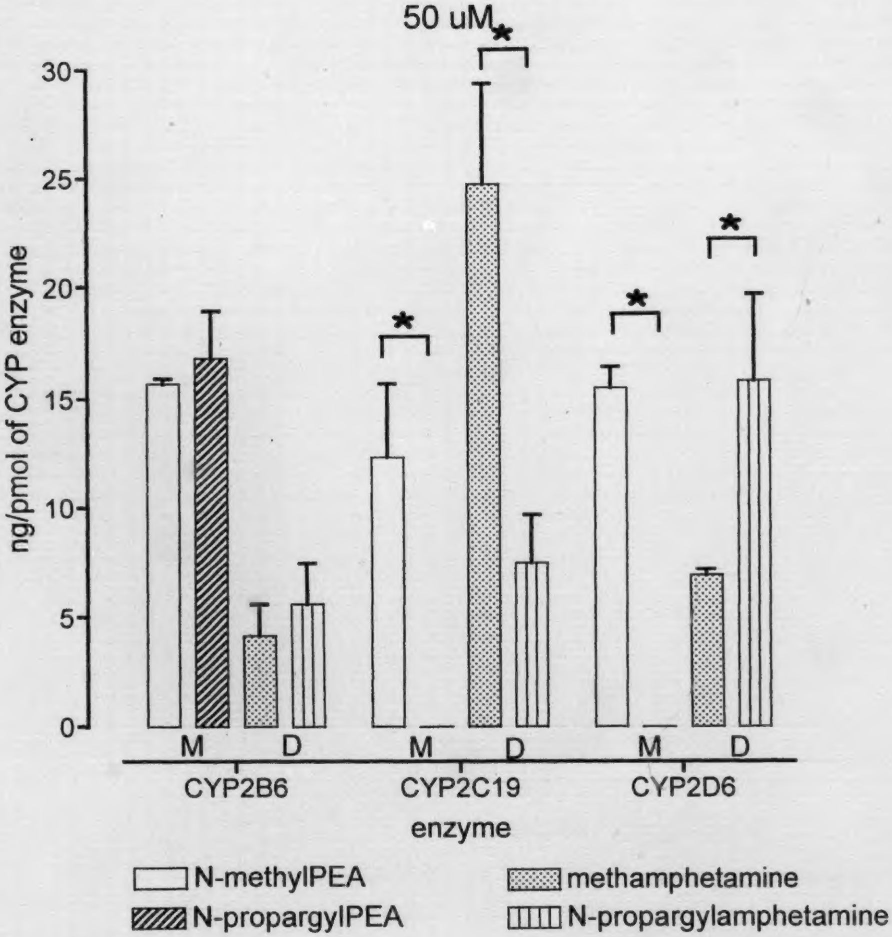


Figure 4.4. Results of incubation of MPPE (M) and (-)-deprenyl (D) (50 μM each) with cDNA-expressed CYP enzymes for 120 minutes. Results are expressed as means ± SEM (N=4). * = $p < 0.05$.

According to our findings reported here, formation of both methamphetamine and N-propargylamphetamine from (-)-deprenyl can indeed be catalyzed by CYP2D6 and CYP2B6. CYP2C19 appears to be important in N-depropargylation of both MPPE and (-)-deprenyl (formation of N-methylPEA and methamphetamine respectively) at 500 µM and 50 µM, but to have no effect on formation of the N-demethylated metabolite of MPPE at either concentration. A small effect was noted on N-propargylamphetamine formation, but the effect was variable and there was not a significant difference between the amounts of this metabolite formed at 50 or 500 µM.

The marked difference in formation of N-demethylated metabolites between the two drugs in the presence of CYP2B6 and CYP2D6 is very interesting. While substantial amounts of N-propragylamphetamine are formed from (-)-deprenyl at both 500 µM and 50 µM in the presence of CYP2D6, under the same conditions no N-propargylPEA is formed. In contrast, at both concentrations, in the presence of CYP2B6, a significantly greater amount of N-demethylated metabolite is formed from MPPE than from an equimolar amount of (-)-deprenyl.

It is clear from our results that MPPE is metabolized similarly to (-)-deprenyl, but with demethylation being greater with MPPE than (-)-deprenyl in the case of CYP2B6 while the opposite effect is evident with CYP2D6. These marked differences are interesting in lieu of the fact that the two drugs differ from one another only in the presence of an additional α-methyl group in (-)-deprenyl. The findings of this study shed some light on the controversy surrounding involvement of CYP enzymes in the metabolism of (-)-deprenyl and emphasize the importance of conducting comprehensive studies on the metabolism of new drugs.

4.5 REFERENCES

Akhondzadeh, S., Tavakolian, R., Davari-Ashtiani, R., Arabgol, F. and Amini, H. (2003) Selegiline in the treatment of attention deficit hyperactivity disorder in children: A double blind and randomized trial. *Prog Neuro-Psychopharmacol* 27:841-845

Bach, M. V., Coutts, R. T. and Baker, G. B. (2000) Metabolism of N,N-dialkylated amphetamines, including deprenyl, by CYP2D6 expressed in a human cell line. *Xenobiotica* 30:297-306

DeLaGarza, V. W. (2003) Pharmacologic treatment of alzheimer's disease: An update. *Am Fam Physician* 68:1365-72

Desta, Z., Zhao, X., Shin, J. G. and Flockhart, D. A. (2002) Clinical significance of the cytochrome P450 2C19 genetic polymorphism. *Clin Pharmacokinet* 41:913-58

Elkashef, A., Fudala, P. J., Gorgon, L., Li, S. H., Kahn, R., Chiang, N., Vocci, F., Collins, J., Jones, K., Boardman, K. and Sather, M. (2006) Double-blind, placebo-controlled trial of selegiline transdermal system (sts) for the treatment of cocaine dependence. *Drug Alcohol Depend*

Faucette, S. R., Hawke, R. L., Lecluyse, E. L., Shord, S. S., Yan, B., Laethem, R. M. and Lindley, C. M. (2000) Validation of bupropion hydroxylation as a selective marker of human cytochrome P450 2B6 catalytic activity. *Drug Metab Dispos* 28:1222-30

Grace, J. M., Kinter, M. T. and Macdonald, T. L. (1994) Atypical metabolism of deprenyl and its enantiomer, (s)-(+)-N,alpha-dimethyl-n-propynylphenethylamine, by cytochrome P450 2D6. *Chem Res Toxicol* 7:286-90

Hesse, L. M., Venkatakrishnan, K., Court, M. H., von Moltke, L. L., Duan, S. X., Shader, R. I. and Greenblatt, D. J. (2000) CYP2B6 mediates the in vitro hydroxylation of bupropion: Potential drug interactions with other antidepressants. *Drug Metab Dispos* 28:1176-1183

Hidestrand, M., Oscarson, M., Salonen, J. S., Nyman, L., Pelkonen, O., Turpeinen, M. and Ingelman-Sundberg, M. (2001) CYP2B6 and CYP2C19 as the major enzymes responsible for the metabolism of selegiline, a drug used in the treatment of parkinson's disease, as revealed from experiments with recombinant enzymes. *Drug Metab Dispos* 29:1480-1484

Kwan, E., Baker, G. B., Shuaib, A., Ling, L. and Todd, K. G. (2000) N-methyl,N-propargyl-2-phenylethylamine (MPPE), an analog of deprenyl, increases neuronal cell survival in thiamin deficiency encephalopathy. *Drug Develop Res* 51:244-252

Mandel, S., Grunblatt, E., Riederer, P., Gerlach, M., Levites, Y. and Youdim, M. B. (2003) Neuroprotective strategies in Parkinson's Disease : An update on progress. *CNS Drugs* 17:729-62

McCann, D. J., Mello, N. K., Negus, S. S., Bergman, J., Forster, M. and Schenk, S. (1999) Selegiline as a potential cocaine treatment medication: Behavioral evaluation in rhesus monkeys and comparisons with other MAO inhibitors in rodents. *Behav Pharmacol* 10:S59-S60

Morita, S., Shimoda, K., Someya, T., Yoshimura, Y., Kamijima, K. and Kato, N. (2000) Steady-state plasma levels of nortriptyline and its hydroxylated metabolites in japanese patients: Impact of CYP2D6 genotype on the hydroxylation of nortriptyline. *J Clin Psychopharmacol* 20:141-9

Olanow, C. W. (2004) The scientific basis for the current treatment of Parkinson's disease. *Ann Rev Med* 55:41-60

Paterson, I. A., Zhang, D., Warrington, R. C. and Boulton, A. A. (1998) R-deprenyl and R-2-heptyl-n-methylpropargylamine prevent apoptosis in cerebellar granule neurons induced by cytosine arabinoside but not low extracellular potassium. *J Neurochem* 70:515-23

Patkar, A. A., Pae, C. U. and Masand, P. S. (2006) Transdermal selegiline: The new generation of monoamine oxidase inhibitors. *CNS Spectr* 11:363-75

Riederer, P. and Lachenmayer, L. (2003) Selegiline's neuroprotective capacity revisited. *J Neural Transm* 110:1273-8

Rittenbach, K., Sloley, B. D., Ling, L., Coutts, R. T., Shan, J. and Baker, G. B. (2005) A rapid, sensitive electron-capture gas chromatographic procedure for analysis of metabolites of N-methyl,N-propargylphenylethylamine, a potential neuroprotective agent. *J Pharmacol Toxicol Methods* 52:373-8

Rittenbach, K. A., Holt, A., Ling, L., Shan, J. and Baker, G. B. (2007) Metabolism of N-methyl,N-propargylphenylethylamine: Studies with human liver microsomes and cDNA expressed cytochrome P450 (CYP) enzymes. *Cell Mol Neurobiol* 27:179-190

Salsali, M., Coutts, R. T. and Baker, G. B. (2000) A rapid electron-capture gas chromatographic procedure for the analysis of p-hydroxymephenytoin. *J Pharmacol Toxicol Methods* 44:461-5

Schapira, A. H. V. and Olanow, C. W. (2004) Neuroprotection in parkinson disease: Mysteries, myths, and misconceptions. *J Am Med Assoc* 291:358-364

Scheinin, H., Anttila, M., Dahl, M. L., Karnani, H., Nyman, L., Taavitsainen, P., Pelkonen, O. and Bertilsson, L. (1998) CYP2D6 polymorphism is not crucial for the disposition of selegiline. *Clin Pharmacol Ther* 64:402-11

Semkova, I., Wolz, P., Schilling, M. and Krieglstein, J. (1996) Selegiline enhances NGF synthesis and protects central nervous system neurons from excitotoxic and ischemic damage. *Eur J Pharmacol* 315:19-30

Sharma, U., Roberts, E. S. and Hollenberg, P. F. (1996) Inactivation of cytochrome P4502B1 by the monoamine oxidase inhibitors r-(-)-deprenyl and clorgyline. *Drug Metab Dispos* 24:669-75

Shin, H. S. (1997) Metabolism of selegiline in humans. Identification, excretion, and stereochemistry of urine metabolites. *Drug Metab Dispos* 25:657-62

Sowa, B. N., Todd, K. G., Tanay, V. A. M. I., Holt, A. and Baker, G. B. (2004) Amine oxidase inhibitors and development of neuroprotective drugs. *Current Neuropharmacol* 2:153-168

Stocchi, F. and Olanow, C. W. (2003) Neuroprotection in Parkinson's Disease: Clinical trials. *Ann Neurol* 53 Suppl 3:S87-97; discussion S97-9

Taavitsainen, P., Anttila, M., Nyman, L., Karnani, H., Salonen, J. S. and Pelkonen, O. (2000) Selegiline metabolism and cytochrome P450 enzymes: In vitro study in human liver microsomes. *Pharmacol Toxicol* 86:215-21

Tabi, T., Magyar, K. and Szoko, E. (2003) Chiral characterization of deprenyl-N-oxide and other deprenyl metabolites by capillary electrophoresis using a dual cyclodextrin system in rat urine. *Electrophoresis* 24:2665-73

Tatton, W. G. (1993) Selegiline can mediate neuronal rescue rather than neuronal protection. *Movement Disorders* 8 Suppl 1:S20-30

Tatton, W. G. and Chalmers-Redman, R. M. (1996) Modulation of gene expression rather than monoamine oxidase inhibition: (-)-deprenyl-related compounds in controlling neurodegeneration. *Neurol* 47:S171-83

Thomas, T. (2000) Monoamine oxidase-B inhibitors in the treatment of Alzheimer's disease. *Neurobiol Aging* 21:343-348

Todd, K. G. and Butterworth, R. F. (1998) Increased neuronal cell survival after l-deprenyl treatment in experimental thiamine deficiency. *J Neurosci Res* 52:240-6

Venkatakrishnan, K., von Moltke, L. L. and Greenblatt, D. J. (1999) Nortriptyline E-10-hydroxylation in vitro is mediated by human CYP2D6 (high affinity) and CYP3A4 (low affinity): Implications for interactions with enzyme-inducing drugs. *J Clin Pharmacol* 39:567-77

Wacher, V. J., Wong, S., Wong, H. T. and Benet, L. Z. (1996) Contribution of CYP3A to selegeline metabolism in rat and human liver microsomes. *Proc. North Am. Meet. Int. Soc. Study Xenobiot., San Diego*

CHAPTER 5

A RAPID, SENSITIVE ELECTRON-CAPTURE GAS CHROMATOGRAPH PROCEDURE FOR ANALYSIS OF METABOLITES OF N-METHYL,N-PROPARGYLPHENTERMINE, A POTENTIAL NEUROPROTECTIVE AGENT[4].

---

[4] A version of this chapter has been submitted for publication. Rittenbach, K.A., 2007. *Journal of Chromatography B*.

5.1 INTRODUCTION

(-)-Deprenyl is currently prescribed for the treatment of PD, as it has been reported to delay the onset of motor symptoms (Heinonen and Myllyla, 1998; Mandel *et al.*, 2003; Shults, 2003; Olanow, 2004). It is also under investigation for use in AD (Birks and Flicker, 2003; DeLaGarza, 2003), attention deficit disorder (Akhondzadeh *et al.*, 2003), the negative symptoms of schizophrenia (Bodkin *et al.*, 2005), cocaine addiction (Yasar *et al.*, 2006) and as an anti-depressant (transdermally) (Patkar *et al.*, 2006). One problem with the use of (-)-deprenyl is that although (-)-deprenyl and one main metabolite (N-propargyl-amphetamine) are potentially neuroprotective (Semkova *et al.*, 1996; Riederer and Lachenmayer, 2003; Stocchi and Olanow, 2003), potentially neurotoxic amphetamine and methamphetamine are also produced during metabolism (Shin, 1997; Taavitsainen *et al.*, 2000) (Figure 5.1).

As part of a structure-activity relationship study, we have synthesized two analogues of deprenyl, namely N-methyl,N-propargylphenylethylamine (MPPE) and N-methyl,N-propargylphentermine (MPPT) which differ from deprenyl structurally by the deletion (MPPE) or addition (MPPT) of a methyl group at the α carbon. Both drugs have been shown, like deprenyl, to be neuroprotective in the DSP-4-induced neurotoxicity model (personal communication). We have now investigated the metabolism of MPPE relative to (-)-deprenyl and found that MPPE is metabolized in a similar fashion to (-)-deprenyl, i.e. by N-depropargyl-ation and N-demethylation, but that the degree of involvement of various CYP enzymes is quite different (Rittenbach *et al.*, 2005; Rittenbach *et al.*, 2007).

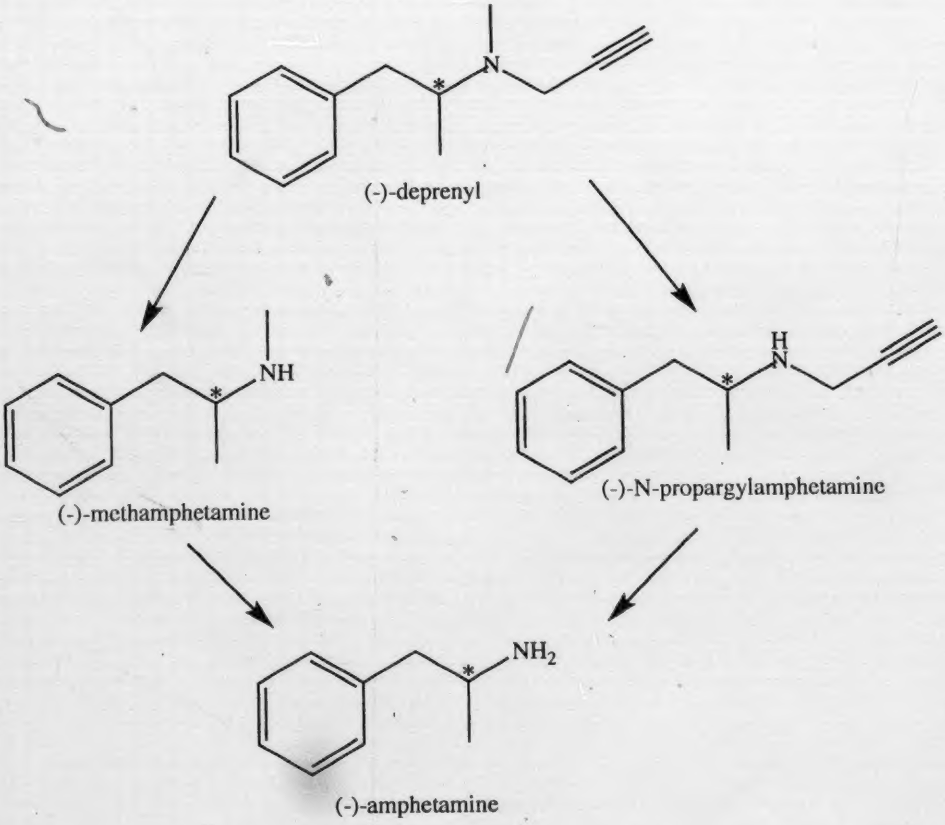

(-)-deprenyl
(-)-methamphetamine
(-)-N-propargylamphetamine
(-)-amphetamine
NH
H
N
NH2

Figure 5.1. Metabolism of deprenyl to methamphetamine, N-propargylamphetamine and amphetamine.

These studies are now going to be extended to MPPT, using the assay described here for simultaneous analysis of the potential metabolites, i.e. N-propargylphentermine, N-methylphentermine and phentermine (Figure 5.2).

To investigate the metabolism of MPPT, a sensitive method for analysis of the proposed metabolites was developed and used in studies with human liver microsomes (HLMs). This method used derivatization with PFBC, a well established technique for the derivatization of amines under aqueous conditions (Delbeke *et al.*, 1983; Fisher *et al.*, 1985; Rao *et al.*, 1986; Baker *et al.*, 1987; Coutts *et al.*, 1987; Salsali *et al.*, 2000; Chou *et al.*, 2004; Xie *et al.*, 2004) (Figure 5.3) followed by electron-capture gas chromatography. This method showed excellent reliability and reproducibility when used to analyze samples from HLMs. The limit of detection (LOD) for N-methylphentermine and N-propargylphentermine were higher than desired and so for low levels of these metabolites a modified procedure was used.

5.2 METHODS

5.2.1 Materials

Phentermine (PHEN) HCl, benzylamine HCl (internal standard, IS), PFBC, glucose-6-phosphate, and β-nicotine adenine dinucleotide phosphate were purchased from Sigma Chemical Company (St. Louis, MO, USA). Glucose-6-phosphate dehydrogenase was purchased from Cederlane Laboratories Limited (Hornby, ON, Canada).

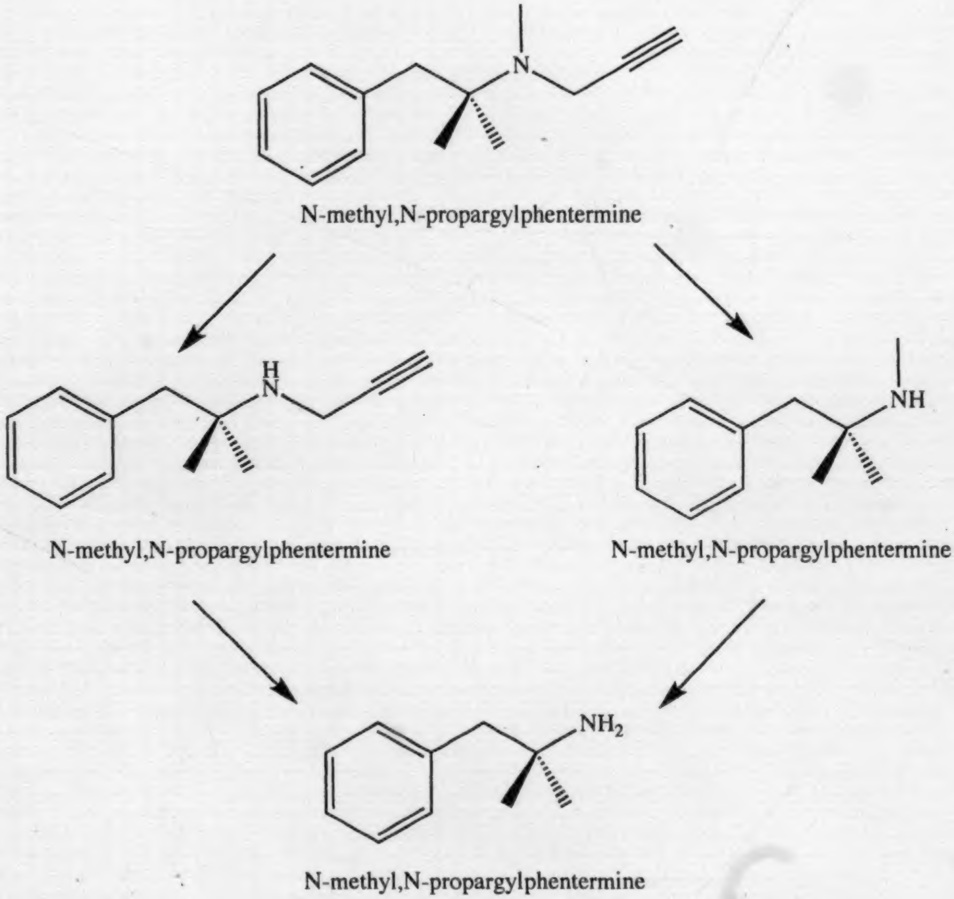

N-methyl,N-propargylphentermine
H
N
N-methyl,N-propargylphentermine
NH
N-methyl,N-propargylphentermine
NH_2
N-methyl,N-propargylphentermine

Figure 5.2. Proposed metabolism of MPPT to N-methylphentermine, N-propargylphentermine and phentermine.

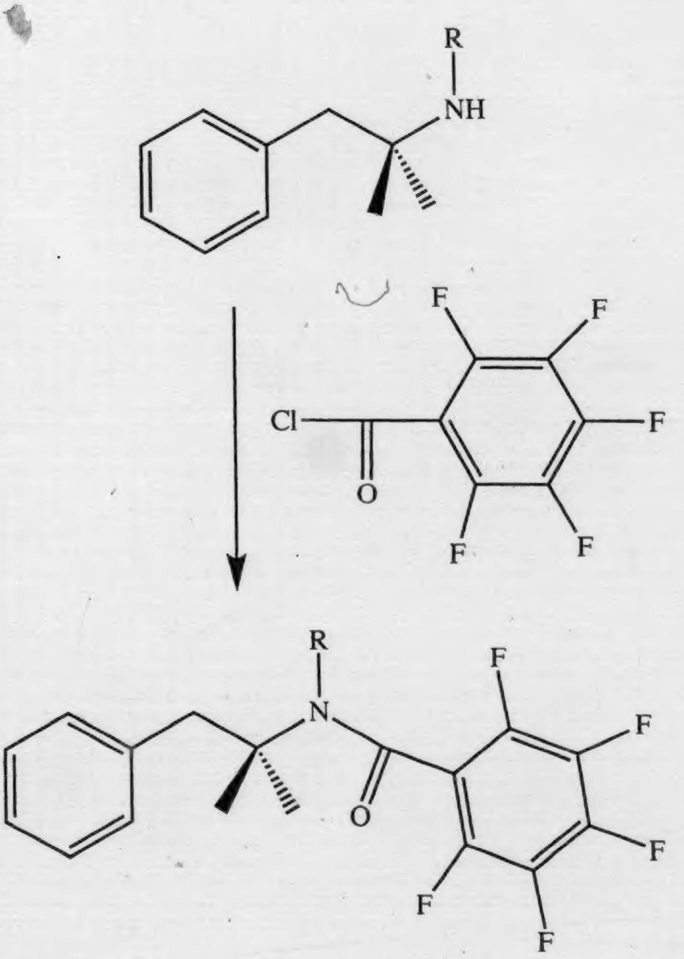

R
NH
F
F
Cl
F
O
F
F
R
N
F
F
O
F
F
F

Figure 5.3. Representation of derivatization of phentermines with PFBC (R=H, CH_3, or CH_2CCH).

MPPT has been previously synthesized in the laboratories of CV Technologies (Edmonton, AB, Canada). For the current study, N-methylPHEN and N-propargylPHEN HCl were synthesized in the University of Alberta Faculty of Pharmacy and Pharmaceutical Sciences (Edmonton, AB, Canada). Both N-methylPHEN and N-propargylPHEN were shown to be stable in aqueous solution at 0-4°C for several days, but fresh dilutions were made from frozen stock solutions for each assay run.

The HLM solution was purchased from Gentest Corporation (Woburn, MA, USA). It was a single donor sample, HG93, with total P450 content of 330 pmol P450/mg protein.

Ethyl acetate, toluene, trisodium phosphate, potassium bicarbonate, potassium carbonate, potassium phosphate monobasic, potassium phosphate dibasic and magnesium chloride were purchased from Fisher Scientific (Mississauga, ON, Canada); all were HPLC or reagent grade and were used without further purification.

5.2.2 Human Liver Microsome Incubations

The microsome incubation medium consisted of 25 μl of an NADPH-generating system (4 mg/ml β-nicotine adenine dinucleotide phosphate, 4 mg/ml glucose-6-phosphate, 3.2 units/ml of glucose-6-phosphate dehydrogenase and 2.6 mg/ml of magnesium chloride in 0.1M potassium phosphate buffer (pH=7.4)), 10 μl of microsomal solution, between 5 μl and 25 μl of MPPT substrate solution (resulting in final concentrations of 50-3000 μM) and sufficient buffer to result in a final volume of 100 μl. The metabolic reaction was allowed to proceed for 20 min in a 37°C water bath. The samples were placed on ice and 50 μl of 25% potassium carbonate solution were added to stop the reaction. Double-distilled water (850 μl) and 75 μl of working IS solution (750 ng) were added and the samples were transferred to screw top tubes.

5.2.3 Sample Preparation, Derivatization and Analysis

To 1 ml aqueous samples of either prepared standards or HLM samples, 300 μL of 10% trisodium phosphate was added. The sample was vortexed briefly and 4 ml of derivatizing reagent (1 ml:1 μl; ethyl acetate:PFBC) were added. After 10 min of vortex mixing, the mixture was centrifuged at 1000 g for 5 min. The upper layer was transferred to a concentration tube and taken to dryness in a Savant Speed-vac evaporator. Toluene (300 μl) was added to the residue and a 2 μl aliquot of this solution was injected for GC-ECD analysis.

To quantitate low levels of N-methylphentermine and N-propargylphentermine this assay was modified. Trials were run that included washing the final toluene layer with water to remove interfering peaks, basifying with sodium phosphate (10%), extraction with Di-(2-ethylhexyl)phosphate (DEHPA) in toluene, and anhydrous derivatization with PFBC after extraction with ethyl acetate. The method that resulted in good repeatability and good sensitivity was extraction followed by anhydrous derivatization. The 1 ml aqueous sample was basified with excess solid potassium bicarbonate, the liquid transferred to a clean tube and 4 ml of ethyl acetate added. After 10 min. of vortex mixing the mixture was centrifuged at 1000 g for 5 min. The upper layer was transferred to a concentration tube and taken to dryness in a Savant speed-vac evaporator. Toluene:PFBC (100 : 1) (200 µl) was added to the residue and heated at 100°C for 15 min.. To this solution, 500 µl of double distilled water was added. This mixture was briefly vortexed and then centrifuged (1000 g for 5 min.) and the toluene layer was transferred to gas-chromatograph vials and a 2 µl aliquot was injected for GC-ECD analysis.

Standard curves consisting of a fixed amount of IS (750 ng) and varying amounts of the three analytes of interest were run in parallel with each assay run. Ratios of analytes to IS were plotted on the x axis against amounts of analyte on the y axis. Amounts of analytes in each sample were determined by comparing ratios of analyte to IS in the sample to those in the standard curve.

5.2.4 GC-ECD Analysis

The analysis was carried out using a Hewlett-Packard HP-6890A gas chromatograph with a micro electron-capture detector with a Ni-63 source. The GC column used was a HP-5 capillary column (25m x 0.32mm i.d., 1.05µm film thickness). The injection temperature was 260°C. The following column temperature ramp was programmed: initial temperature of 120°C (held for 1.0 min), increasing at 30°C/min to 210°C (held for 5 min), increasing at 5°C/min to 230°C, and increasing at 30°C/min to 300°C (held for 10 min). The carrier gas was helium, with argon-methane (90-10) used as the makeup gas at the detector.

5.2.5 GC-MS analysis

GC-MS analysis was performed on an Agilent 6890 Series gas chromatograph coupled to an Agilent 5973 Network Mass Selective Detector. Analysis was conducted in the electron-impact (electron ionization, EI) mode.

5.3 RESULTS

The procedure is rapid and gives peaks with excellent chromatographic properties (Figure 5.4). Structures of the derivatives were confirmed by GC-MS, and the mass fragmentaion patterns are shown in Figures 5.5-5.7. The recovery, intra-assay reliability and inter-assay reliability of the method were determined. Recovery was measured as the percent of a given concentration of analyte extracted from the aqueous medium compared to the same concentration derivatized in the organic phase without extraction. Recoveries were 104.6%, 92.6% and 88.6% for PHEN, N-methylPHEN and N-propargylPHEN, respectively (Table 5.1). Intra-assay C.V.s were calculated by extracting 5 samples of the same concentration and comparing the analyte to IS ratio; this was done at five different concentrations for each compound (250 ng/ml, 500 ng/ml, 1000 ng/ml, 1500 ng/ml and 2000 ng/ml). For all three compounds the intra-assay C.V.s were less than 4% at all five of the concentrations (Table 5.2). Interassay C.V.s were calculated using the same five concentrations from 5 curves from different days and values are shown in Table 5.3. Phentermine interassay C.V.s are less than 5% for all five concentrations; N-methylPHEN and N-

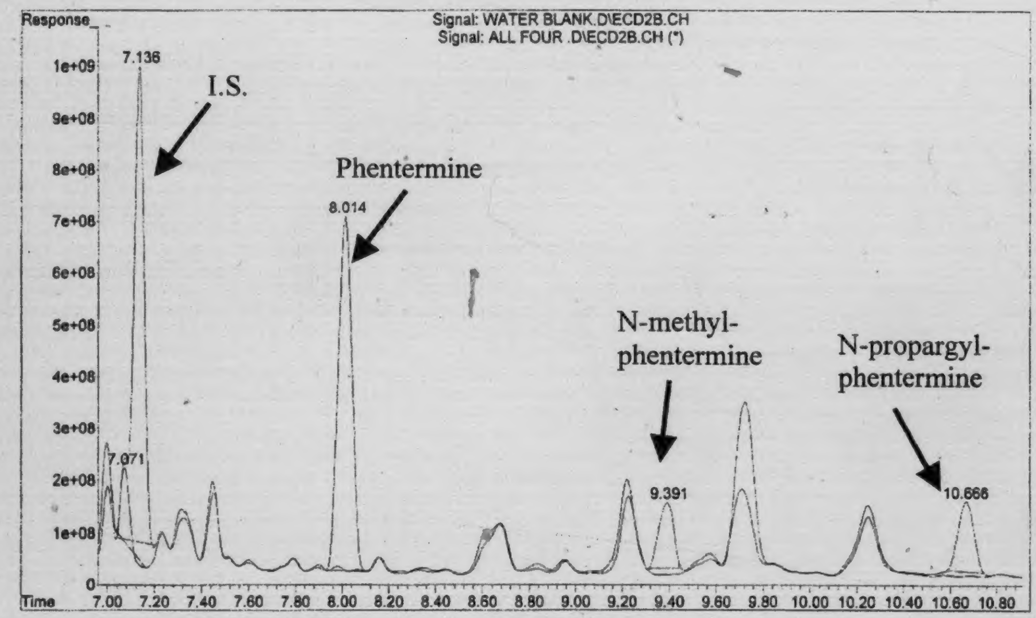


Figure 5.4. Representative trace of compounds of interest.

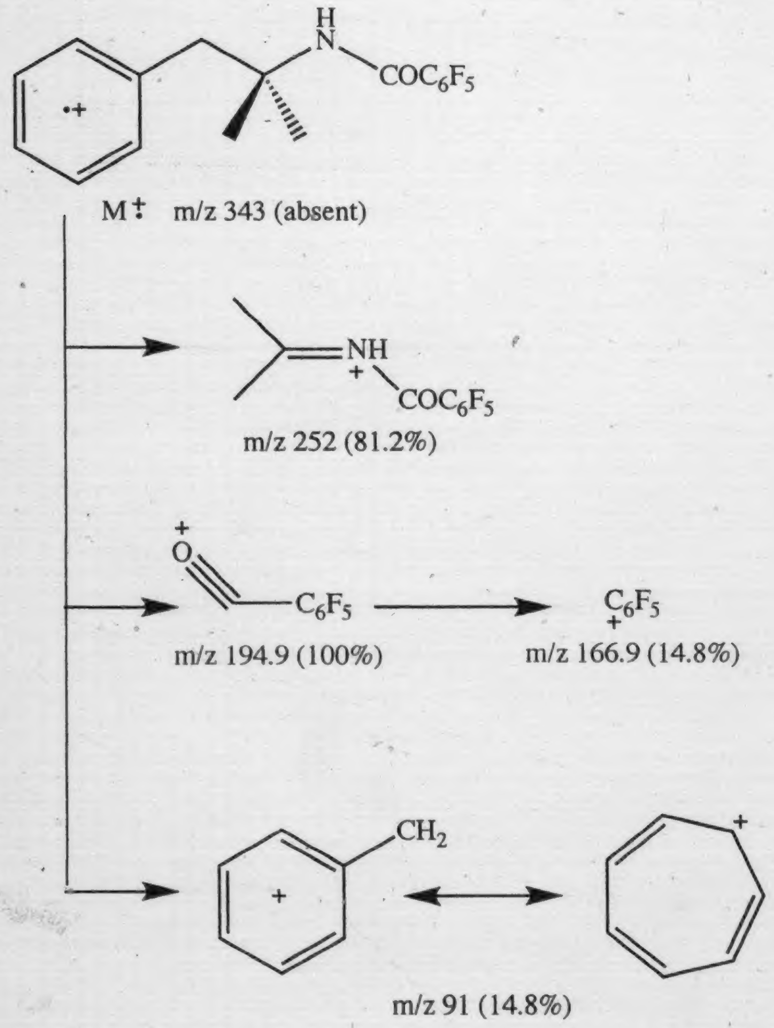
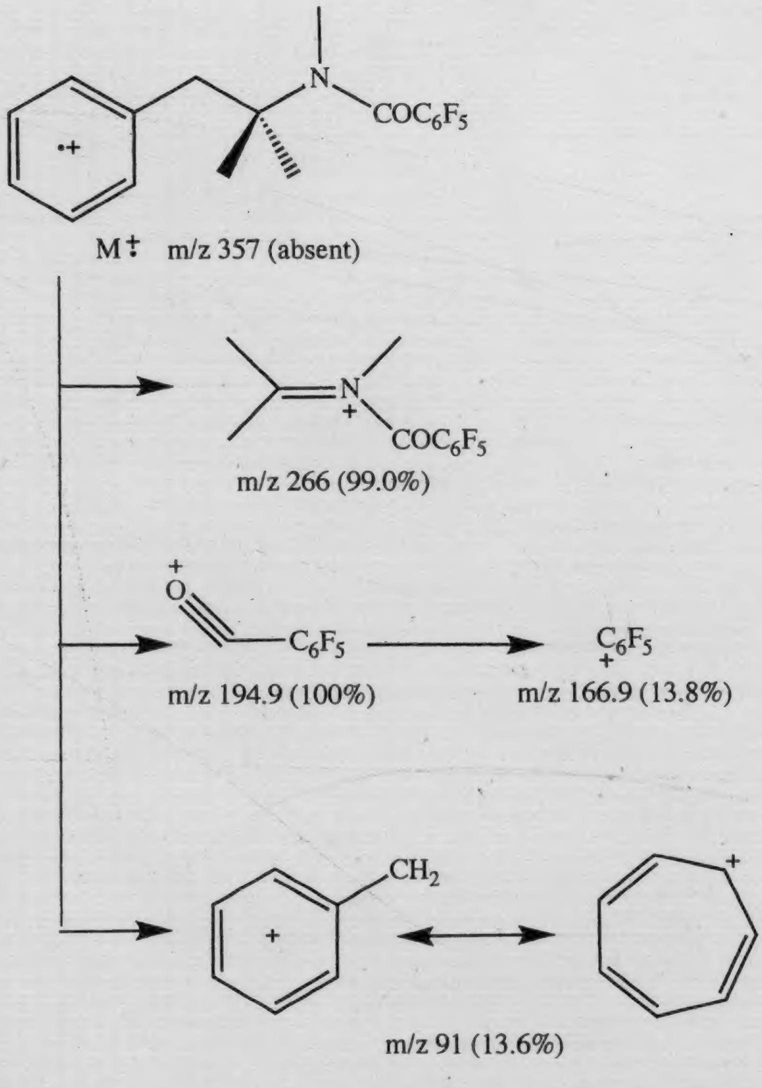

H
N
COC_6F_5
•+
M+• m/z 343 (absent)
NH
+
COC_6F_5
m/z 252 (81.2%)
+
O
C_6F_5
m/z 194.9 (100%)
C_6F_5
+
m/z 166.9 (14.8%)
CH_2
+
+
m/z 91 (14.8%)

Figure 5.5. Mass spectral fragmentation of phentermine derivatized with PFBC. Percentages represent percent abundance relative to the base peak.



N
COC$_6$F$_5$
•+
M$^{+\bullet}$ m/z 357 (absent)
N
+
COC$_6$F$_5$
m/z 266 (99.0%)
+
O
C$_6$F$_5$
m/z 194.9 (100%)
C$_6$F$_5$
+
m/z 166.9 (13.8%)
CH$_2$
+
+
m/z 91 (13.6%)

Figure 5.6. Mass spectral fragmentation of N-methylphentermine derivatized with PFBC. Percentages represent percent abundance relative to the base peak.

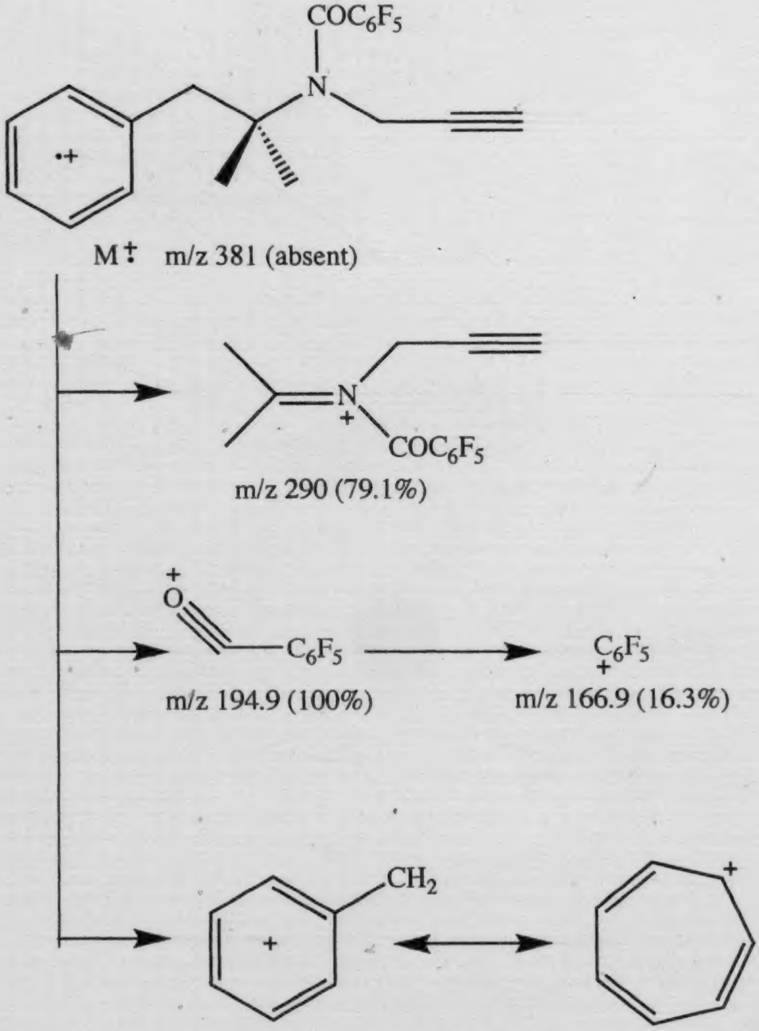

COC$_6$F$_5$
N
•+
M$^{+\cdot}$ m/z 381 (absent)
N
+
COC$_6$F$_5$
m/z 290 (79.1%)
+
O
C$_6$F$_5$
m/z 194.9 (100%)
C$_6$F$_5$
+
m/z 166.9 (16.3%)
CH$_2$
+
+
m/z 91 (19.1%)

Figure 5.7. Mass spectral fragmentation of N-propargylphentermine derivatized with PFBC. Percentages represent percent abundance relative to the base peak.

Concentration (ng/ml)	Phentermine	N-methylPHEN	N-propargylPHEN
250	111.7	102.7	93.8
500	98.9	90.1	86.7
1000	108.7	86.2	83.8
1500	103.3	98.9	91.2
2000	100.4	85.1	87.7

Table 5.1. Recovery data, values shown are percentages of the analyte of extracted samples compared to directly derivatized samples (N=5).

Concentraton (ng/ml)	Phentermine	N-methylPHEN	N-propargylPHEN
250	2.37	2.36	1.74
500	2.16	3.82	1.72
1000	1.49	0.98	0.42
1500	1.67	0.85	0.99
2000	3.16	1.87	2.15

Table 5.2. Intra-assay reliability C.V. data, values shown are percentages [SD/mean x 100%] (N=5).

propargylPHEN are somewhat higher (averaging 5.8% and 11.3% respectively). These results showed that this method is robust and reliable.

The method is linear for the concentrations investigated with correlation coefficients of $r^2 > 0.99$ routinely obtained. The slope, intercepts, standard error of the slope and intercepts and limits of detection are shown in Table 5.4, note that the best fit for PHEN was always a quadratic equation. The curves contained greater than 10 points, from 2 ng/ml to 2000 ng/ml for PHEN, 10 ng/ml to 2500 ng/ml for N-methylPHEN and 50 ng/ml to 2750 ng/ml for N-propargylPHEN.

5.4 DISCUSSION

Extractive derivatization with PFBC under aqueous conditions has been shown to be useful in the analysis of many endogenous and exogenous compounds containing amine and/or phenol groups (Delbeke, *et al.*, 1983; Fisher, *et al.*, 1985; Rao, *et al.*, 1986; Baker, *et al.*, 1987; Coutts, *et al.*, 1987; Salsali, *et al.*, 2000; Chou, *et al.*, 2004; Xie, *et al.*, 2004). This study shows that the method can be used conveniently to analyze these three structurally similar compounds (PHEN, N-methylPHEN and N-propargylPHEN) simultaneously, which has not been shown previously, though had to be modified for the lowest concentrations of N-methylPHEN and N-propargylPHEN.

There are several published methods for the analysis of PHEN (Palmer *et al.*, 2000; Kaddoumi *et al.*, 2001a; Namera *et al.*, 2002; Kaddoumi *et al.*, 2003; Apollonio *et al.*, 2006). However, many use instruments that are not common in most labs, such as tandem mass spectrometry (MS-MS)

Concentraton (ng/ml)	Phentermine	N-methylPHEN	N-propargylPHEN
250	2.02	11.62	11.53
500	2.51	8.76	18.49
1000	1.87	4.29	5.85
1500	3.11	2.72	10.23
2000	4.03	2.00	10.63

Table 5.3. Inter-assay reliability C.V. data, values shown are percentages [(SD/mean) x 100%] (N=5).

Compound	Line		R^2	LOD
	Slope (mean ± sem)	Intercept (mean ± sem)		ng/ml
Phentermine	$-9.4\text{x}10^{-7} \pm 4.6\text{x}10^{-7}\ Y^2 +$ $1.4\text{x}10^{-3} \pm 2.9\text{x}10^{-4}$	0.12 ± 0.028	0.992	1
N-methyl phentermine	$8.3\text{x}10^{-5} \pm 2.6\text{x}10^{-6}$	$3.4\text{x}10^{-3} \pm 3.6\text{x}10^{-4}$	0.991	15*
N-propargyl phentermine	$6.7\text{x}10^{-5} \pm 1.6\text{x}10^{-6}$	$1.1\text{x}10^{-2} \pm 3.1\text{x}10^{-3}$	0.991	15*

Table 5.4. Standard curve data for phentermine, N-methylphentermine and N-propargylphentermine. (N=5) * modified method. LOD = limit of detection.

(De Leenheer *et al.*, 2001), capillary electrophoresis (Ku *et al.*, 1999), and headspace solid phase micro extraction (Namera, *et al.*, 2002). Others use high-performance liquid chromatography (Kaddoumi *et al.*, 2001b). A concern in several of the published methods is interference between amphetamines and phentermine (Klette *et al.*, 2005; Apollonio *et al.*, 2006). The method presented here is rapid, sensitive, separates amphetamine and phentermine and uses an economical separation and detection system.

This method has now been used to study the formation of these three compounds from MPPT using HLM systems (Figure 5.8). This has shown that N-methylPHEN and N-propargylPHEN are metabolites of this potential neuroprotective agent; ongoing work will further elucidate the metabolic pathways and the enzymes involved.

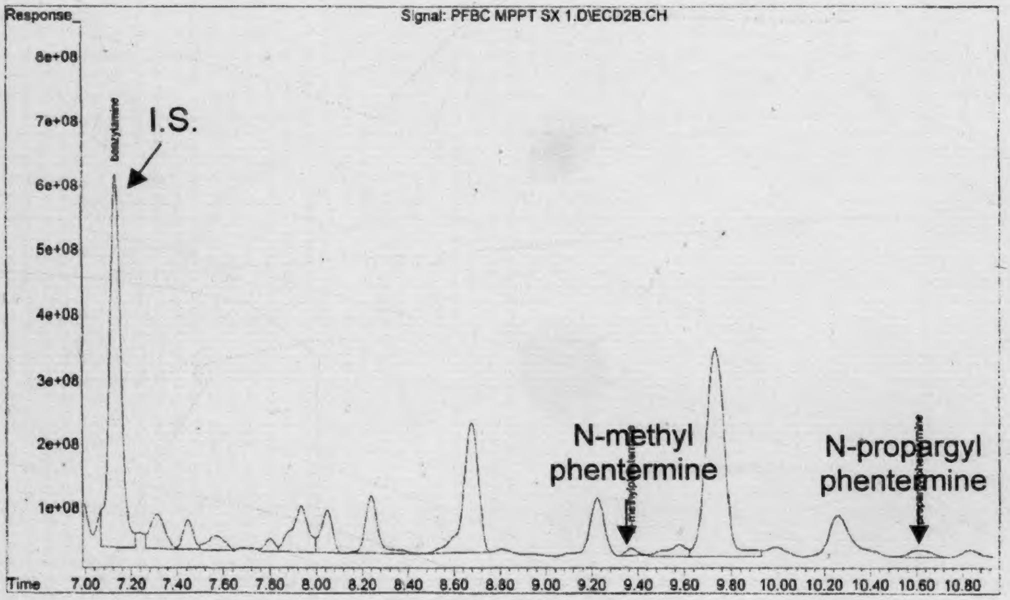


Figure 5.8. Representative GC trace of the derivatized products of 500 μM MPPT incubated with HLMs for 2 hours.

5.5 REFERENCES:

Akhondzadeh, S., Tavakolian, R., Davari-Ashtiani, R., Arabgol, F. and Amini, H. (2003) Selegiline in the treatment of attention deficit hyperactivity disorder in children: A double blind and randomized trial. *Prog Neuro-Psychopharmacol* 27:841-845

Apollonio, L. G., Pianca, D. J., Whittall, I. R., Maher, W. A. and Kyd, J. M. (2006) A demonstration of the use of ultra-performance liquid chromatography-mass spectrometry [uplc/ms] in the determination of amphetamine-type substances and ketamine for forensic and toxicological analysis. *J Chromatogr B* 836:111-115

Baker, G. B., Wong, J. T., Coutts, R. T. and Pasutto, F. M. (1987) Simultaneous extraction and quantitation of several bioactive amines in cheese and chocolate. *J Chromatogr* 392:317-31

Birks, J. and Flicker, L. (2003) Selegiline for Alzheimer's disease. *Cochrane Database System Review* CD000442

Bodkin, J. A., Siris, S. G., Bermanzohn, P. C., Hennen, J. and Cole, J. O. (2005) Double-blind, placebo-controlled, multicenter trial of selegiline augmentation of antipsychotic medication to treat negative symptoms in outpatients with schizophrenia. *Am J Psychiatry* 162:388-390

Chou, S.-L., Yang, M.-H., Ling, Y.-C. and Giang, Y.-S. (2004) Gas chromatography-isotope dilution mass spectrometry preceded by liquid-liquid extraction and chemical derivatization for the determination of ketamine and norketamine in urine. *J Chromatogr B* 799:37-50

Coutts, R. T., Torok-Both, G. A., Tam, Y. K., Chu, L. V. and Pasutto, F. M. (1987) Acetylation and pentafluorobenzoylation of lidocaine metabolites in aqueous solution and identification of derivatives by combined gas chromatography/mass spectrometry. *Biomed Environ Mass Spectrom* 14:173-82

De Leenheer, A. P., Decaestecker, T., E., D. L., Clauwaert, K., Bouche, M. P., Lambert, W., Van Bocxlaer, J., Piette, M., Van den Eeckhout, E. and Van Peteghem, C. (2001) Technical Note: Fatal 4-MTA Intoxication: Development of a Liquid Chromatographic-Tandem Mass Spectrometric Assay for Multiple Matrices. *J Anal Toxicol* 25:705-710

DeLaGarza, V. W. (2003) Pharmacologic treatment of Alzheimer's disease: An update. *Am Fam Physician* 68:1365-72

Delbeke, F. T., Debackere, M., Jonckheere, J. A. and De Leenheer, A. P. (1983) Pentafluorobenzoyl derivatives of doping agents. I. Extractive benzoylation and gas chromatography with electron-capture detection of primary and secondary amines. *J Chromatogr* 273:141-9

Fisher, D. H., Adams, J. and Giese, R. W. (1985) Trace derivatization of cytosine with pentafluorobenzoyl chloride and dimethyl sulfate. *Environ Health Perspect* 62:67-71

Heinonen, E. H. and Myllyla, V. (1998) Safety of selegiline (deprenyl) in the treatment of Parkinson's disease. *Drug Safety* 19:11-22

Kaddoumi, A., Kubota, A., Nakashima, M. N., Takahashi, M. and Nakashima, K. (2001a) High performance liquid chromatography with UV detection for the simultaneous determination of sympathomimetic amines using 4-(4,5-diphenyl-1*h*imidazole-2-yl)benzoyl chloride as a label. *Biomed Chromatogr* 15:379-388

Kaddoumi, A., Nakashima, M. N., Maki, T., Matsumura, Y., Nakamura, J. and Nakashima, K. (2003) Liquid chromatography studies on the pharmacokinetics of phentermine and fenfluramine in brain and blood microdialysates after intraperitoneal administration to rats. *J Chromatogr B* 791:291-303

Kaddoumi, A., Nakashima, M. N. and Nakashima, K. (2001b) Fluorometric determination of dl-fenfluramine, dl-norfenfluramine and phentermine in plasma by achiral and chiral high-performance liquid chromatography. *J Chromatogr B Biomed Sci Appl* 763:79-90

Klette, K. L., Jamerson, M. H., Morris-Kukoski, C. L., Kettle, A. R. and Snyder, J. J. (2005) Rapid simultaneous determination of amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine, 3,4-methylenedioxymethamphetamine, and 3,4-methylenedioxyethylamphetamine in urine by fast gas chromatography-mass spectrometry. *J Anal Toxicol* 29:669-74

Ku, Y. R., Chang, Y. S., Wen, K. C. and Ho, L. K. (1999) Analysis and confirmation of synthetic anorexics in adulterated traditional chinese medicines by high-performance capillary electrophoresis. *J Chromatogr A* 848:537-43

Mandel, S., Grunblatt, E., Riederer, P., Gerlach, M., Levites, Y. and Youdim, M. B. (2003) Neuroprotective strategies in Parkinson's Disease : An update on progress. *CNS Drugs* 17:729-62

Namera, A., Yashiki, M. and Kojima, T. (2002) Automated headspace solid-phase microextraction and in-matrix derivatization for the determination of amphetamine-related drugs in human urine by gas chromatography-mass spectrometry. *J Chromatogr Sci* 40:19-25

Olanow, C. W. (2004) The scientific basis for the current treatment of Parkinson's Disease. *Ann Rev Med* 55:41-60

Palmer, R. B., Kim, N.-H. and Dasgupta, A. (2000) Simultaneous determination of fenfluramine and phentermine in urine using gas chromatography mass spectrometry with pentafluoropropionic anhydride derivatization. *Ther Drug Monit* 22:418-422

Patkar, A. A., Pae, C. U. and Masand, P. S. (2006) Transdermal selegiline: The new generation of monoamine oxidase inhibitors. *CNS Spectr* 11:363-75

Rao, T. S., Baker, G. B. and Coutts, R. T. (1986) Pentafluorobenzenesulfonyl chloride as a sensitive reagent for the rapid gas chromatographic analysis of tranylcypromine in tissues and body fluids. *Biochem Pharmacol* 35:1925-8

Riederer, P. and Lachenmayer, L. (2003) Selegiline's neuroprotective capacity revisited. *J Neural Transm* 110:1273-8

Rittenbach, K., Sloley, B. D., Ling, L., Coutts, R. T., Shan, J. and Baker, G. B. (2005) A rapid, sensitive electron-capture gas chromatographic procedure for analysis of metabolites of N-methyl,N-propargylphenylethylamine, a potential neuroprotective agent. *J Pharmacol Toxicol Methods* 52:373-8

Rittenbach, K. A., Holt, A., Ling, L., Shan, J. and Baker, G. B. (2007) Metabolism of N-methyl,N-propargylphenylethylamine: Studies with human liver microsomes and cDNA expressed cytochrome P450(CYP) enzymes. *Cell Mol Neurobiol* 27:179-190

Salsali, M., Coutts, R. T. and Baker, G. B. (2000) A rapid electron-capture gas chromatographic procedure for the analysis of *p*-hydroxymephenytoin. *Journal of Pharmacological and Toxicological Methods* 44:461-465

Semkova, I., Wolz, P., Schilling, M. and Krieglstein, J. (1996) Selegiline enhances ngf synthesis and protects central nervous system neurons from excitotoxic and ischemic damage. *Eur J Pharmacol* 315:19-30

Shin, H. S. (1997) Metabolism of selegiline in humans. Identification, excretion, and stereochemistry of urine metabolites. *Drug Metab Dispos* 25:657-62

Shults, C. W. (2003) Treatments of Parkinson Disease: Circa 2003. *Arch Neurol* 60:1680-1684

Stocchi, F. and Olanow, C. W. (2003) Neuroprotection in Parkinson's disease: Clinical trials. *Ann Neurol* 53 Suppl 3:S87-97; discussion S97-9

Taavitsainen, P., Anttila, M., Nyman, L., Karnani, H., Salonen, J. S. and Pelkonen, O. (2000) Selegiline metabolism and cytochrome P450 enzymes: In vitro study in human liver microsomes. *Pharmacol Toxicol* 86:215-21

Xie, S., Aspromonte, J., Balla, A., Sershen, H., Javitt, D. C. and Cooper, T. B. (2004) Sensitive and simple gas chromatographic-mass spectrometric determination for amphetamine in microdialysate and ultrafiltrate samples. *J Chromatogr B* 805:27-31

Yasar, S., Gaal, J., Panlilio, L. V., Justinova, Z., Molnar, S. V., Redhi, G. H. and Schindler, C. W. (2006) A comparison of drug-seeking behavior maintained by d-amphetamine, l-deprenyl (selegiline), and d-deprenyl under a second-order schedule in squirrel monkeys. *Psychopharmacology (Berl)* 183:413-21

CHAPTER 6

METABOLISM OF N-METHYL,N-PROPARGYLPHENTERMINE: STUDIES WITH HUMAN LIVER MICROSOMES AND cDNA EXPRESSED CYTOCHROME P450 (CYP) ENZYMES

6.1 INTRODUCTION

(-)-Deprenyl has been shown to be neuroprotective in a large variety of both *in vivo* and *in vitro* models of neurotoxicity (Tatton, 1993; Semkova *et al.*, 1996; Tatton and Chalmers-Redman, 1996; Paterson *et al.*, 1998; Todd and Butterworth, 1998; Tatton *et al.*, 2000; Ebadi *et al.*, 2002; Riederer and Lachenmayer, 2003; Magyar and Szende, 2004; Sowa *et al.*, 2004). As a selective, irreversible monoamine-oxidase (MAO) –B inhibitor, (-)-deprenyl was originally investigated as a potential antidepressant that would not produce the "cheese effect" when administered with food containing tyramine or other sympathomemetic substances. It was generally shown to be a poor antidepressant except at higher doses where it inhibited MAO-A as well as MAO-B, but recent reports suggest that it may be useful at lower doses for treating depression and cocaine dependence when administered transdermally (Elkashef *et al.*, 2006; Patkar *et al.*, 2006).

The main clinical use of (-)-deprenyl is in PD, where it reduces the reliance on L-DOPA and can slow deterioration (Heinonen and Myllyla, 1998). There are also studies showing clinical efficacy in AD (DeLaGarza, 2003) and recent work demonstrates a direct action on amyloid fibrils which is a novel mechanism of action (DeLaGarza, 2003; Ono *et al.*, 2006). However, inhibition of MAO does not seem to be the primary mechanism of action for (-)-deprenyl's neuroprotective/neurorescue effects and the mechanism of action appears to involve complex actions or factors affecting apoptosis (Youdim *et al.*, 2006).

One major metabolite of (-)-deprenyl is N-propargylamphetamine, which has shown neuroprotection (Mytilineou *et al.*, 1997; Mytilineou *et al.*, 1998; Maruyama and Naoi, 1999). The other two major metabolites, amphetamine and methamphetamine, however, can be neurotoxic even in low concentrations (Am *et al.*, 2004).

A novel analogue of (-)-deprenyl, synthesized in our laboratory, that shows MAO-B inhibition and efficacy in preventing DSP-4 induced reduction in hippocampal noradrenaline (personal communication) is N-methyl,N-propargylphentermine (MPPT). If metabolism of MPPT parallels that of (-)-deprenyl, it would be predicted to produce N-methylphentermine (N-methyl-PHEN), N-propargylphentermine(N-methylPHEN) and finally phentermine (PHEN) (Figure 6.1). Phentermine is a stimulant that has been shown to damage dopaminergic neurons, but it is not as potent as the amphetamines produced by (-)-deprenyl (McCann *et al.*, 1998; Callahan *et al.*, 2000). The damage from PHEN was only seen at the highest concentrations tested and was species-dependent (Glazer, 2001). Further, PHEN has been used safely in the United States since 1959 (Glazer, 2001; Kim *et al.*, 2006).

In order to study the metabolism of MPPT, a rapid, sensitive method for the detection of these three metabolites was developed in our laboratory (see chapter 5). Preliminary studies using this method demonstrated that PHEN and its N-alkyl derivatives are indeed metabolites of MPPT (see chapter 5 of this thesis). In this article we present data from a fluorometric screening

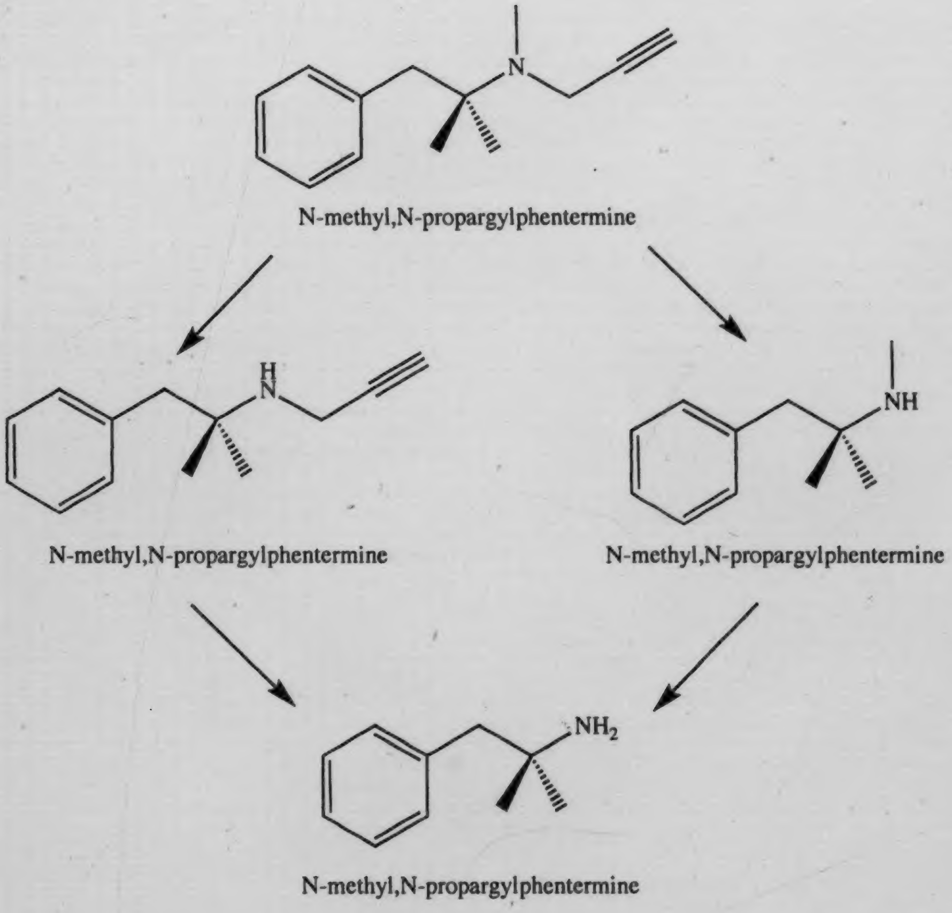

N
N-methyl,N-propargylphentermine
H
N
N-methyl,N-propargylphentermine
NH
N-methyl,N-propargylphentermine
NH2
N-methyl,N-propargylphentermine

Figure 6.1. Theorized metabolism of N-methyl,N-propargylphentermine to N-methylphentermine, N-propargylphentermine and finally phentermine.

procedure which identifies the interactions between MPPT and cDNA-expressed CYP enzymes, and a kinetic analysis of MPPT metabolism by HLMs.

6.2 MATERIALS AND METHODS

6.2.1 Materials

Phentermine (PHEN) HCl, benzylamine HCl (internal standard, IS), pentafluorobenzoyl chloride (PFBC), glucose-6-phosphate, and β-nicotine adenine dinucleotide phosphate were purchased from Sigma Chemical Company (St. Louis, MO, USA). Glucose-6-phosphate dehydrogenase was purchased from Cederlane Laboratories Limited (Hornby, ON, Canada).

MPPT has been previously synthesized in the laboratories of CV Technologies (Edmonton, AB, Canada). For the current study, N-methylPHEN and N-propargylPHEN were synthesized in the Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta (Edmonton, AB, Canada) by Dr. E.E. Knaus. Both N-methylPHEN and N-propargylPHEN were shown to be stable in aqueous solution at 0-4°C for several days, but fresh dilutions were made from frozen stock solutions for each assay run.

The HLM solution was purchased from Gentest Corporation (Woburn, MA, USA). It was a single donor sample, HG93, with total P450 content of 330 pmol P450/mg protein.

The baculovirus-expressed human CYP1A2, 2B6, 2C19, 2D6, 3A4, 3-cyano-7-ethoxycoumarin, 7-ethoxy-4-(trifluoromethyl)-coumarin, 3-[2-(N,N-diethyl-N-methylammonium)ethyl]-7-hydroxy-4-methylcoumarin and 7-

benzyloxy-4-(trifluoromethyl)-coumarin were purchased from Gentest Corporation (Woburn, MA, USA). Activity results were 25 pmol product per (mg protein x min.) for CYP2B6 using the (S)-mephenytoin N-demethylase assay, 200 pmol product per (mg protein x min.) for CYP2C19 using the (S)-mephenytoin 4'-hydroxylase, and for CYP2D6 63 pmol product per (mg protein x min.) of bufuralol 1'-hydroxylase was inhibited by 1 µM quinidine.

Ethyl acetate, toluene, trisodium phosphate, potassium bicarbonate, potassium carbonate, potassium phosphate monobasic, potassium phosphate dibasic and magnesium chloride were purchased from Fisher Scientific (Mississauga, ON, Canada); all were HPLC or reagent grade and were used without further purification.

6.2.2 Fluorogenic screen for interaction of MPPT with CYP enzymes

MPPT (0.1 nM to 1 mM) was preincubated with the NADPH-generating solution for 10 min. at 37°C. The cDNA-expressed CYP enzyme and corresponding fluorogenic substrate were then added to the wells and incubated at 37°C (1A2 for 15 min., 2B6, 2C19, and 2D6 for 30 min., 2C9 and 3A4 for 60 min.). Enzyme activity was terminated by the addition of 75 µl of a 1:4 (v/v) solution of Tris (0.5 M) /acetonitrile. The fluorogenic substrates yield a fluorescent product upon metabolism by the appropriate CYP enzyme (Table 6.1). Product-derived fluorescence was measured in a Molecular Devices Gemini XPS plate reader. Velocity data obtained in the presence of MPPT at various concentrations were fitted with the non-linear regression facility of GraphPad

Substrate	3-cyano-7-ethoxy-coumarin	7-ethyoxy-4-(trifluoro-methyl)-coumarin	3-[2-(N,N-diethyl-N-methyl-ammonium) ethyl]-7-hydroxy-4-methylcoumarin	7-benzyloxy-4-(trifluoro-methyl)-coumarin
CYP	1A2, 2C19	2B6	2D6	3A4
Product	3-cyano-7-hydroxy-coumarin	7-hydroxy-4-trifluoro-methyl-coumarin	3-[2-(N,N-diethylamino)ethyl]-7-hydroxy-4-methylcoumarin hydrochoride	7-hydroxy-4-trifluoro-methyl-coumarin
Product l_{ex}/l_{em}	410_{ex}/ 460_{em} nm	409_{ex} /530_{em} nm	390_{ex} /460_{em} nm	409_{ex} /530_{em} nm

Table 6.1. Fluorogenic CYP substrates and excitation and emission wavelengths used in the present studies.

Prism (GraphPad Software Inc., San Diego, CA, USA) to an equation for linear competitive inhibition (Equation 6.1).

$$v = \frac{Top + (Bottom - Top)}{1 + 10^{\log_{10}\left(K_i\left(1+\frac{[S]}{K_M}\right)\right) - \log_{10}[MPPE]}} \qquad \text{[Equation 6.1]}$$

In this equation, Top and Bottom refer to the horizontal upper and lower limits of the sigmoidal curve, respectively, and [S] and K_M are values relating to metabolism of the fluorogenic substrate. Fitting sigmoidal curves with this equation allows direct determination of a K_i value (inhibitor dissociation constant) for the inhibition of CYP activity by MPPT, and thus an estimate for K_M, should MPPT act as a substrate for the CYP enzyme of interest. In order to determine whether or not MPPT was indeed metabolized, further studies were completed.

6.2.3 Human liver microsome incubations

The microsome incubation medium consisted of 25 μl of an NADPH-generating system (4 mg/ml β-nicotine adenine dinucleotide phosphate, 4 mg/ml glucose-6-phosphate, 3.2 units/ml of glucose-6-phosphate dehydrogenase and 2.6 mg/ml of magnesium chloride in 0.1M potassium phosphate buffer (pH=7.4)), 10 μl of microsomal solution, between 5 μl and 25 μl of MPPT substrate solution (resulting in final concentrations of 50-3000 μM) and sufficient buffer to result in a final volume of 100 μl. The metabolic reaction was allowed to proceed for 20 min in a 37°C water bath. The samples were placed on ice and 50 μl of 25%

potassium carbonate solution were added to stop the reaction. Double-distilled water (850 μl) and 75 μl of working IS solution (750 ng) were added and the samples were transferred to screw top tubes. In a second experiment 4.05 μl of N-methylPHEN (1 mg/ml or 1 mg/10 ml) or 4.53 μl of N-propargylPHEN (1 mg/ml or 1 mg/10 ml) were added instead of the MPPT, resulting in 25 μM or 250 μM respectively, this incubation was run for 60 min. at 37°C, and the resulting PHEN concentrations measured.

6.2.4 Sample preparation, derivatization and analysis

The analysis of samples has been described elsewhere (see chapter 5 of this thesis). Briefly, the aqueous samples were basified and shaken with a solution of PFBC in ethyl acetate. The oganic phase was retained and taken to dryness using a Savant Speed-vac evaporator. The residue was dissolved in toluene, and an aliquot of this solution was analyzed in a gas chromatograph equipped with a capillary column and an electron-capture detector. This analysis was also used for the quantification of N-propargylamphetamine in (-)-deprenyl incubations. Amphetamine and methamphetamine were analyzed using the method described in Rittenbach *et al.* (2005). All three curves were linear with R^2 values of 0.9897 for amphetamine (N=12), 0.9869 for methamphetamine (N=14) and 0.9881 for N-propargylamphetamine (N=14).

6.2.5 Studies on metabolism of MPPT by cDNA-expressed CYP enzymes

The microsome incubation medium consisted of 25 μl of an NADPH-generating solution, 10 μl of microsomal solution (cDNA expressed 2B6, 2C19 or 2D6), 10.0 μl of MPPT (1 mg/ml or 1 mg/10 ml) or 9.3 μl of deprenyl solution (1 mg/ml or 1 mg/ 10 ml) resulting in final concentrations of 50 and 500 μM respectively, and sufficient buffer to bring the final volume to 100 μl. Previous studies established the role of these enzymes in the metabolism of (-)-deprenyl and so the (-)-deprenyl samples acted as positive controls (Rittenbach and Baker, 2007).

6.3 RESULTS

In the fluorometric screen MPPT interacted with the c-DNA-expressed CYP enzymes 2B6, 2C19 and 2D6 (Figure 6.2) with dissociation constants (K_i values with 95% confidence range) of 3.1 μM (2.7 to 3.4), 1.6 μM (1.2 to 2.2), and 8.1 μM (7.4 to 8.7) respectively. There was no interaction with 1A2 or 2C9 at the concentrations studied, and the K_i for 3A4 was two orders of magnitude higher than for the three enzymes studied further. All of the compounds tested (MPPT, N-methylPHEN, N- propargylPHEN and PHEN) inhibited CYP2D6, but the only other inhibition was by N-methylPHEN which inhibited CYP2B6 and 2C19.

Studies measuring the amount of N-methylPHEN, N-propargylPHEN and PHEN produced upon incubation of MPPT (50 and 500 μM) with CYP 2B6, 2C19 and 2D6 elucidated which enzymes catalyzed the production of these

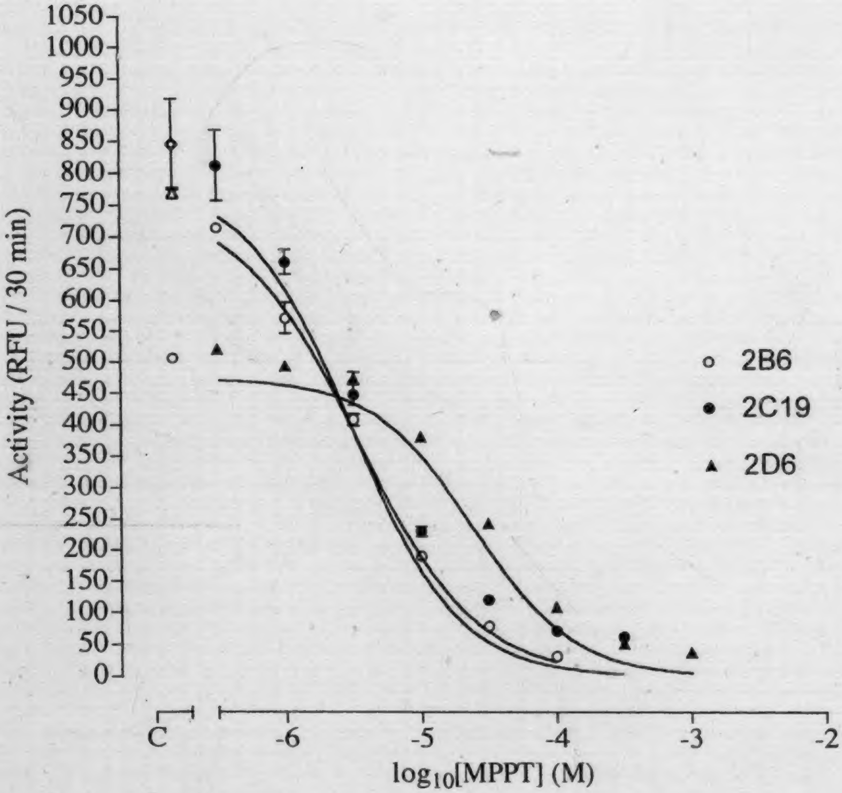


Figure 6.2. Inhibition by MPPE of metabolism of fluorogenic substrates by CYP2B6 (○), CYP2C19 (●) and CYP 2D6 (▲) (N=3). C = controls. Results are expressed as means ± SEM; where SEM bars are not shown, they were less than 5% of the mean.

metabolites. CYP 2B6 catalyzed the demethylation and depropargylation at both concentrations, while CYP 2C19 catalyzed the depropargylation at both concentrations and demethylation only at the higher concentration. CYP 2D6 did not catalyze the production of either of these N-dealkylated products. (-)-Deprenyl was included as a positive control in these studies and measurable amounts of methamphetamine and N-propargylamphetamine were formed from (-)-deprenyl, as has been reported elsewhere (Grace *et al.*, 1994; Taavitsainen *et al.*, 2000; Hidestrand *et al.*, 2001; Salonen *et al.*, 2003; Rittenbach and Baker, 2007).

MPPT (50 μM to 3 mM) was incubated with an individual donor human liver microsome panel, and the resulting phentermine, N-methylPHEN, and N-propargylPHEN were measured at 20 minutes (Figure 6.3). An earlier time course study had established linear production of the metabolites for longer than 20 minutes. The cDNA-expressed enzyme systems had shown that both N-methylPHEN and N-propargylPHEN formation are catalyzed by two enzymes each with an associated K_M and V_{max} value. Accordingly, data were fitted to Equation 6.2, for metabolism of a single substrate by two enzymes, with the nonlinear regression facility of GraphPad Prism.

$$V = \frac{V_{max1}[S]}{K_{M1} + [S]} + \frac{V_{max2}[S]}{K_{M2} + [S]} \qquad \text{[Equation 6.2]}$$

Nonlinear regression analysis with this equation, while giving reasonable curves, did not allow the determination of K_M and V_{max} values with confidence because of

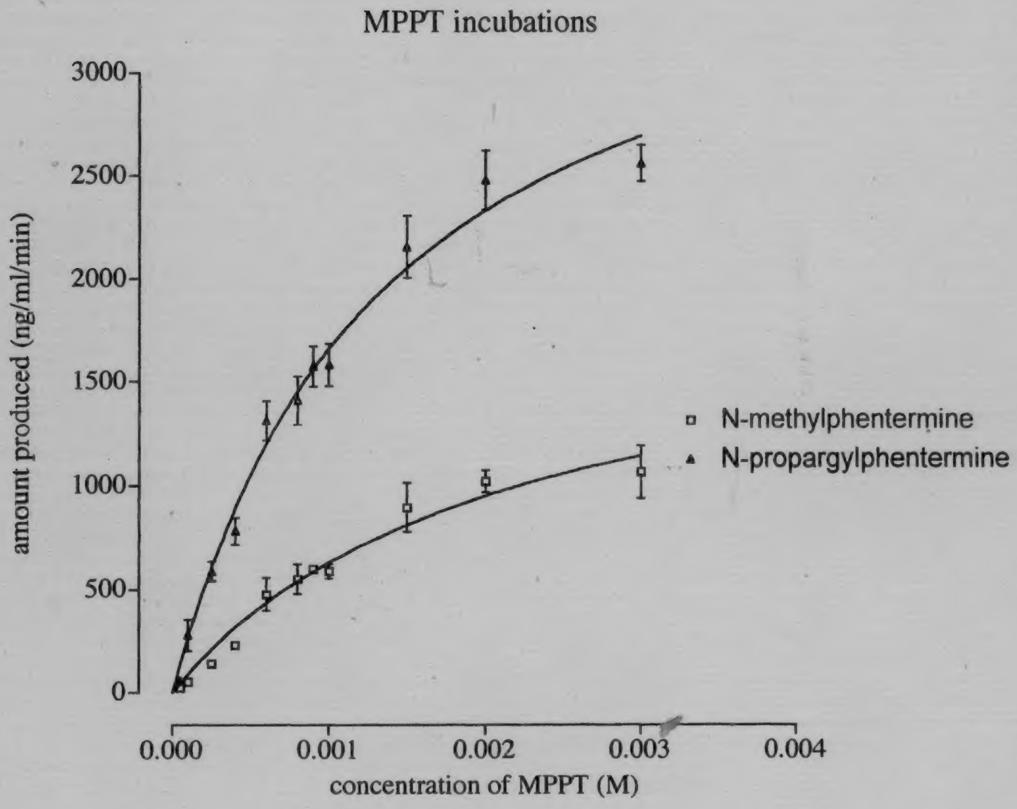


Figure 6.3. Kinetic plots for CYP-mediated generation of N-methylPHEN and N-propargylPHEN by human liver microsomes (N=4).

the variability in the points contributes to further variability in the curves. For illustrative purposes the curves are shown in figure 6.3.

Incubation of N-methylphentermine and N-propargylphentermine with HLMs resulted in measurable amounts of phentermine at both 25 μM and 250 μM (Figure 6.4) demonstrating that these two metabolites of MPPT are further metabolized to phentermine.

6.4 DISCUSSION

In the field of neuropsychiatry many patients are subjected to polypharmaceutical regimens which increases the need for knowledge of metabolism to prevent clinically relevant drug-drug interactions (Harvey and Preskorn, 1996; Nemeroff *et al.*, 1996; Kennedy *et al.*, 2001). MPPT is of interest because it has been shown to be neuroprotective in an animal model that (-)-deprenyl demonstrates neuroprotection in (unpublished communication). The present study demonstrates that MPPT is metabolized similarly to (-)-deprenyl, resulting in N-methylPHEN, N-propargylPHEN and PHEN by human liver microsomes.

The fluorescence study described in this paper does not indicate if the compound that inhibits formation of the fluorescent product (in this case the drugs under investigation, MPPT, N-methylPHEN, N-propargylPHEN and PHEN) is a substrate for the enzymes. However, it does provide data indicating an interaction that can be further investigated. Initial studies showed interactions with CYP2D6 by all of the compounds; the only other interactions were MPPT and N-

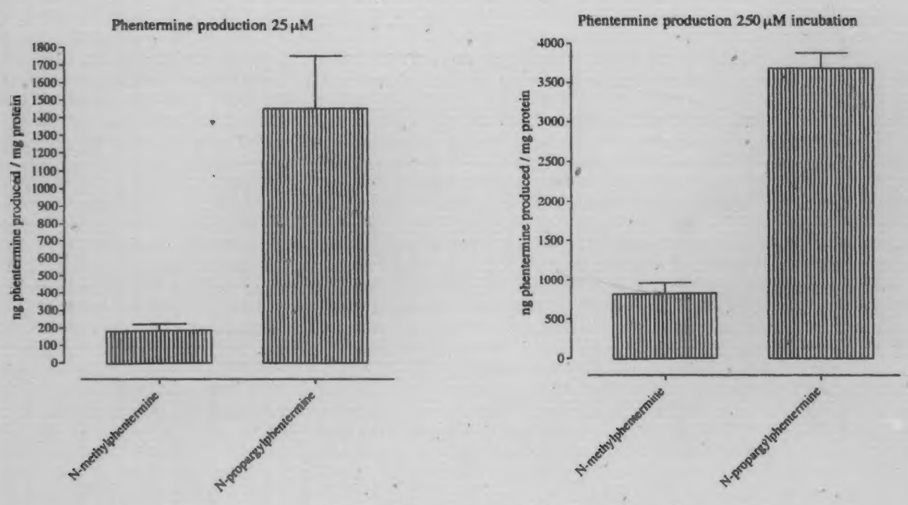


Figure 6.4. Amount of phentermine produced from incubations of N-methylphentermine and N-propargylphentermine at 25 and 250 μM for 60 min. (N=4).

methylPHEN with CYP2B6 and 2C19. The rank order of interactions for MPPT (the compound of primary interest) were CYP2C19, 2B6 and 2D6 (from strongest to weakest). Further studies showed that 2C19 and 2B6 both N-depropargylated and N-demethylated MPPT, while CYP2D6 did not catalyze the formation of either of these metabolites. It is, however, also possible that any N-methylPHEN and N-propargylPHEN formed by CYP2D6 was metabolized further during the incubation.

Knowing that two enzymes catalyzed the formation of both N-methyPHEN and N-propargylPHEN, the kinetic data collected with HLMs had to be fitted to a two-enzyme curve. The resulting curves did not calculate the kinetic constants with reasonable confidence. Further, any N-methylPHEN produced in these incubations would competitively inhibit the further metabolism of MPPT, making interpretation of this data more complex. Further studies with the primary metabolites would be necessary to calculate the kinetic constants of MPPT metabolism with HLM with confidence.

6.5 REFERENCES:

Am, O. B., Amit, T. and Youdim, M. B. H. (2004) Contrasting neuroprotective and neurotoxic actions of respective metabolites of anti-Parkinson drugs rasagiline and selegiline. *Neurosci Lett* 355:169-172

Callahan, B. T., Yuan, J. and Ricaurte, G. A. (2000) Fluoxetine increases the anorectic and long-term dopamine-depleting effects of phentermine. *Synapse (New York, N.Y* 38:471-6

DeLaGarza, V. W. (2003) Pharmacologic treatment of Alzheimer's disease: an update. *Am Fam Physician* 68:1365-72

Ebadi, M., Sharma, S., Shavali, S. and El Refaey, H. (2002) Neuroprotective actions of selegiline. *J Neurosci Res* 67:285-9

Elkashef, A., Fudala, P. J., Gorgon, L., Li, S. H., Kahn, R., Chiang, N., Vocci, F., Collins, J., Jones, K., Boardman, K. and Sather, M. (2006) Double-blind, placebo-controlled trial of selegiline transdermal system (STS) for the treatment of cocaine dependence. *Drug Alcohol Depend* 85:191-7

Glazer, G. (2001) Long-term pharmacotherapy of obesity 2000: a review of efficacy and safety. *Archives Int Med* 161:1814-24

Grace, J. M., Kinter, M. T. and Macdonald, T. L. (1994) Atypical metabolism of deprenyl and its enantiomer, (S)-(+)-N,alpha-dimethyl-N-propynylphenethylamine, by cytochrome P450 2D6. *Chem Res Toxicol* 7:286-90

Harvey, A. T. and Preskorn, S. H. (1996) Cytochrome P450 enzymes: Interpretation of their interactions with selective serotonin reuptake inhibitors Part 1. *J Clin Psychopharmacol* 16:273-285

Heinonen, E. H. and Myllyla, V. (1998) Safety of selegiline (deprenyl) in the treatment of Parkinson's disease. *Drug Safety* 19:11-22

Hidestrand, M., Oscarson, M., Salonen, J. S., Nyman, L., Pelkonen, O., Turpeinen, M. and Ingelman-Sundberg, M. (2001) CYP2B6 and CYP2C19 as the major enzymes responsible for the metabolism of selegiline, a drug used in the treatment of Parkinson's disease, as revealed from experiments with recombinant enzymes. *Drug Metab Dispos* 29:1480-1484

Kennedy, S. H., Lam, R. W., Cohen, N. L., Ravindran, A. V. and Group, t. C. D. W. (2001) Clinical guidelines for the treatment of depression disorders IV. Medications and other biological treatments. *Can J Psychiatry* 46:38S-58S

Kim, K. K., Cho, H. J., Kang, H. C., Youn, B. B. and Lee, K. R. (2006) Effects on weight reduction and safety of short-term phentermine administration in Korean obese people. *Yonsei Med J* 47:614-25

Magyar, K. and Szende, B. (2004) (-)-Deprenyl, a selective MAO-B inhibitor, with apoptotic and anti-apoptotic properties. *NeuroToxicol* 25:233-242

Maruyama, W. and Naoi, M. (1999) Neuroprotection by (-)-deprenyl and related compounds. *Mech Ageing Dev* 111:189-200

McCann, U. D., Yuan, J. and Ricaurte, G. A. (1998) Neurotoxic effects of +/- fenfluramine and phentermine, alone and in combination, on monoamine neurons in the mouse brain. *Synapse* 30:239-46

Mytilineou, C., Leonardi, E. K., Radcliffe, P., Heinonen, E. H., Han, S. K., Werner, P., Cohen, G. and Olanow, C. W. (1998) Deprenyl and desmethylselegiline protect mesencephalic neurons from toxicity induced by glutathione depletion. *J Pharmacol Exp Ther* 284:700-6

Mytilineou, C., Radcliffe, P. M. and Olanow, C. W. (1997) L-(-)-desmethylselegiline, a metabolite of selegiline [L-(-)-deprenyl], protects mesencephalic dopamine neurons from excitotoxicity in vitro. *J Neurochem* 68:434-6

Nemeroff, C. B., DeVane, C. L. and Pollock, B. G. (1996) Newer antidepressants and the Cytochrome P450 system. *Am J Psychiatry* 153:311-320

Ono, K., Hasegawa, K., Naiki, H. and Yamada, M. (2006) Anti-Parkinsonian agents have anti-amyloidogenic activity for Alzheimer's beta-amyloid fibrils in vitro. *Neurochem Int* 48:275-85

Paterson, I. A., Zhang, D., Warrington, R. C. and Boulton, A. A. (1998) R-deprenyl and R-2-heptyl-N-methylpropargylamine prevent apoptosis in cerebellar granule neurons induced by cytosine arabinoside but not low extracellular potassium. *J Neurochem* 70:515-23

Patkar, A. A., Pae, C. U. and Masand, P. S. (2006) Transdermal selegiline: the new generation of monoamine oxidase inhibitors. *CNS Spectr* 11:363-75

Riederer, P. and Lachenmayer, L. (2003) Selegiline's neuroprotective capacity revisited. *J Neural Transm* 110:1273-8

Rittenbach, K. and Baker, G. B. (2007) Involvement of cytochromes P450 2D6, 2B6 and 2C19 in the metabolism of (-)-deprenyl and N-methyl,N-propargylphenylethylamine. *Drug Metab Lett* 1:97-100

Rittenbach, K., Sloley, B. D., Ling, L., Coutts, R. T., Shan, J. and Baker, G. B. (2005) A rapid, sensitive electron-capture gas chromatographic procedure for analysis of metabolites of N-methyl,N-propargylphenylethylamine, a potential neuroprotective agent. J Pharmacol Toxicol Methods 52:373-8

Salonen, J. S., Nyman, L., Boobis, A. R., Edwards, R. J., Watts, P., Lake, B. G., Price, R. J., Renwick, A. B., Gomez-Lechon, M.-J., Castell, J. V., Ingelman-Sundberg, M., Hidestrand, M., Guillouzo, A., Corcos, L., Goldfarb, P. S., Lewis, D. F. V., Taavitsainen, P. and Pelkonen, O. (2003) Comparative studies on the cytochrome P450-associated metabolism and interaction potential of selegiline between human liver-derived in vitro systems. *Drug Metab Dispos* 31:1093-1102

Semkova, I., Wolz, P., Schilling, M. and Krieglstein, J. (1996) Selegiline enhances NGF synthesis and protects central nervous system neurons from excitotoxic and ischemic damage. *Eur J Pharmacol* 315:19-30

Sowa, B. N., Todd, K. G., Tanay, V. A. M. I., Holt, A. and Baker, G. B. (2004) Amine oxidase inhibitors and development of neuroprotective drugs. *Curr Neuropharmacol* 2:153-168

Taavitsainen, P., Anttila, M., Nyman, L., Karnani, H., Salonen, J. S. and Pelkonen, O. (2000) Selegiline metabolism and cytochrome P450 enzymes: in vitro study in human liver microsomes. *Pharmacol Toxicol* 86:215-21

Tatton, W. G. (1993) Selegiline can mediate neuronal rescue rather than neuronal protection. *Movement Disord* 8 Suppl 1:S20-30

Tatton, W. G. and Chalmers-Redman, R. M. (1996) Modulation of gene expression rather than monoamine oxidase inhibition: (-)-deprenyl-related compounds in controlling neurodegeneration. *Neurol* 47:S171-83

Tatton, W. G., Chalmers-Redman, R. M., Elstner, M., Leesch, W., Jagodzinski, F. B., Stupak, D. P., Sugrue, M. M. and Tatton, N. A. (2000) Glyceraldehyde-3-phosphate dehydrogenase in neurodegeneration and apoptosis signaling. *J Neural Transm Suppl* 77-100

Todd, K. G. and Butterworth, R. F. (1998) Increased neuronal cell survival after L-deprenyl treatment in experimental thiamine deficiency. *J Neurosci Res* 52:240-6

Youdim, M. B., Edmondson, D. and Tipton, K. F. (2006) The therapeutic potential of monoamine oxidase inhibitors. *Nature Rev* 7:295-309

CHAPTER 7

INVOLVEMENT OF CYTOCHROME P450 2D6, 2B6, AND 2C19 IN THE METABOLISM OF (-)-DEPRENYL AND N-METHYL,N-PROPARGYLPHENTERMINE

7.1 INTRODUCTION

(-)-Deprenyl (N-methyl,N-propargylamphetamine) is being investigated for use in many disorders, including AD (DeLaGarza, 2003), attention deficit disorder (Akhondzadeh *et al.*, 2003), cocaine addiction (McCann, D.J. *et al.*, 1999), but see also (Elkashef *et al.*, 2006), and transdermally as an antidepressant (Patkar *et al.*, 2006). The only current approved clinical use for this irreversible monoamine oxidase (MAO)-B inhibitor is in PD, where it can delay the onset of motor symptoms and delay the need for L-dopa therapy (Thomas, 2000; Mandel *et al.*, 2003; Riederer and Lachenmayer, 2003; Olanow, 2004; Schapira and Olanow, 2004). (-)-Deprenyl has demonstrated neuroprotective and/or neurorescue effects in numerous *in vitro* and *in vivo* experiments (Tatton, 1993; Semkova *et al.*, 1996; Tatton and Chalmers-Redman, 1996; Paterson *et al.*, 1998; Todd and Butterworth, 1998; Stocchi and Olanow, 2003; Sowa *et al.*, 2004).

In humans, the primary metabolites of (-)-deprenyl are methamphetamine and N-propargylamphetamine (Shin, 1997) (see Figure 7.1). Other, minor, identified metabolites include hydroxylated compounds and the more recently identified deprenyl-N-oxide (Tabi *et al.*, 2003). The enzymes involved in these metabolic pathways have been debated for years.

The earliest reports demonstrated involvement of cytochrome P450 (CYP) 2D6 (Grace *et al.*, 1994; Sharma *et al.*, 1996) and CYP3A (Wacher *et al.*, 1996) in the metabolism of (-)-deprenyl. Soon thereafter, however, Scheinin *et al.* reported that CYP2D6 polymorphism did not affect (-)-deprenyl metabolism,

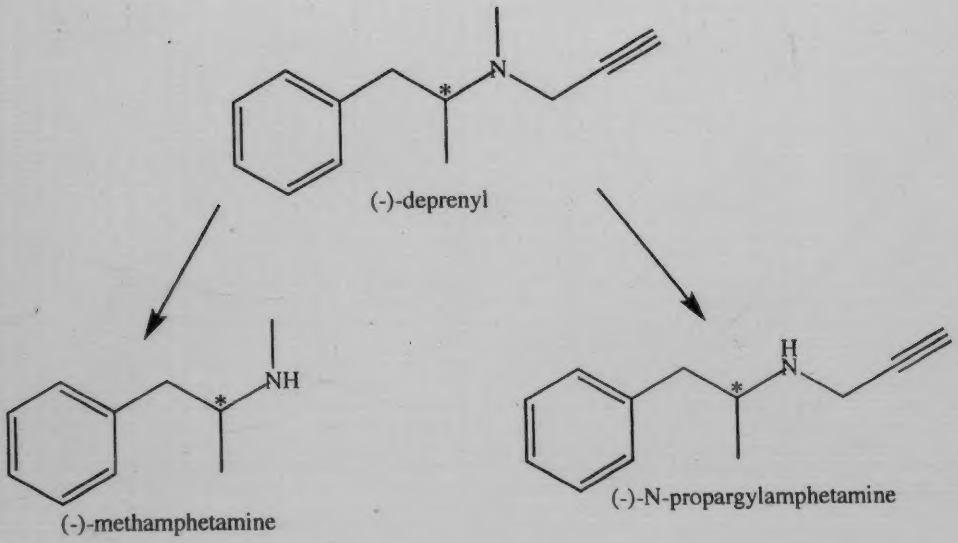

(-)-deprenyl
N
*
NH
(-)-methamphetamine
H
N
(-)-N-propargylamphetamine

Figure 7.1. Metabolism of (-)-deprenyl to methamphetamine, and N-propargylamphetamine.

implying that CYP2D6 was not of primary importance in the elimination of (-)-deprenyl (Scheinin *et al.*, 1998).

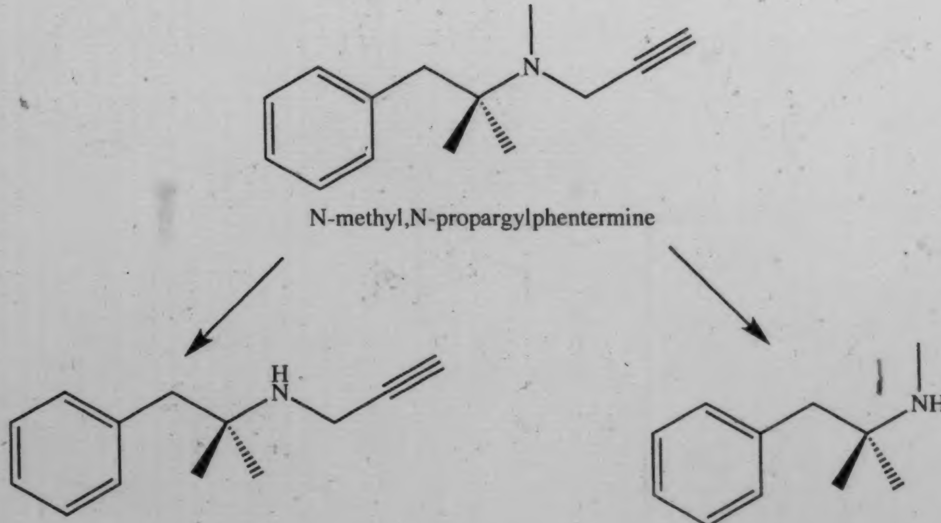
More contradictory reports were published in 2000. One study showed involvement of CYPs 1A2, 3A4, and 2C19, but not 2D6 (Taavitsainen *et al.*, 2000), while another indicated CYP2D6 involvement again (Bach *et al.*, 2000). The study by Taavitsainen *et al.* reported that CYP1A2 catalyzed depropargylation but not demethylation and that CYP3A4 catalyzed both pathways using known inhibitors of CYP enzymes and human liver microsomes (HLM) panels (Taavitsainen, *et al.*, 2000). The method used in the study published by Bach *et al.* differed significantly because they utilized a fortified expressed CYP2D6 preparation.

A more recent study, using human cDNA expressed in yeast cells, investigated the involvment of CYPs 1A1, 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1 and 3A4 in the metabolism of (-)-deprenyl (Hidestrand *et al.*, 2001). They reported that CYP2B6 and CYP2C19 were the major enzymes responsible for the metabolism of (-)-deprenyl (Hidestrand, *et al.*, 2001). This is supported by our recent work also (Rittenbach and Baker, 2007).

Differences reported in the metabolism of (-)-deprenyl may be explained in part by the use of different experimental systems and natural compensation by alternative pathways present in HLM preparations and in human subjects such as 2D6 poor metabolizers.

N-Methyl,N-propargylphentermine (MPPT) is a structural analogue of (-)-deprenyl that differs from deprenyl only in the addition of a second methyl group in the alpha position of the side chain. Like (-)-deprenyl, MPPT is neuroprotective in the DSP-4 hippocampal noradrenaline depletion model (unpublished communication). We have shown that the metabolism of MPPT parallels that of (-)-deprenyl, resulting in formation of N-methylphentermine (N-methylPHEN) and N-propargylphentermine (N-propargylPHEN) (Figure 7.2). These two primary metabolites are further metabolized to phentermine, a noradrenergic anorectic stimulant that has been used safely in the United States since 1959 (Glazer, 2001; Kim *et al.*, 2006). While it has been demonstrated to damage dopaminergic neurons, this damage was only reported at the highest doses studied and further was species-dependent as mice demonstrate decreases while rats do not (McCann, U. D. *et al.*, 1998; Callahan *et al.*, 2000).

A screen with fluorogenic substrates was used by us to elucidate the CYP enzymes with the strongest interactions with MPPT from CYP1A2, 2B6, 2C9, 2C19, 2D6 and 3A4. Studies of metabolite formation using the CYP enzymes identified as interacting with MPPT were completed. The present paper reports on the comparison of (-)-deprenyl and MPPT metabolism by cDNA-expressed CYPs 2B6, 2C19 and 2D6 in experiments conducted under the same conditions, i.e. equimolar amounts of (-)-deprenyl and MPPE with the same amounts of enzyme for the same incubation time.



N-methyl,N-propargylphentermine

N-methyl,N-propargylphentermine

N-methyl,N-propargylphentermine

Figure 7.2. Metabolism of MPPT to N-methylphentermine, and N-propargylphentermine.

7.2 METHODS

7.2.1 Materials

Phentermine (PHEN) HCR, benzylamine HCl (internal standard, IS), glucose-6-phosphate (G6P), (-)-deprenyl, pentafluorobenzoyl chloride (PFBC) and β-nicotine adenine dinucleotide phosphate (NADP) were purchased from Sigma Chemical Company (St. Louis, MO, USA). N-MethylPHEN and N-propargylPHEN were synthesized by Dr. E.E. Knaus in the Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta (Edmonton, AB, Canada). MPPT was synthesized in the laboratories of CV Technologies (Edmonton, AB, Canada). Glucose-6-phosphate dehydrogenase (G6PD) was purchased from Cederlane Laboratory Limited (Hornby, ON, Canada). Baculovirus-expressed CYPs 2B6, 2C19, and 2D6 were purchased from Gentest Corporation (Woburn, MA, USA).

Ethyl acetate, toluene, potassium carbonate, potassium bicarbonate, potassium phosphate monobasic, potassium phosphate dibasic, sodium triphosphate and magnesium chloride were purchased from Fisher Scientific (Mississauga, ON, Canada); all were HPLC or reagent grade and were used without further purification.

7.2.2 Studies on metabolism of MPPT using cDNA-expressed CYP enzymes

The microsome incubations were run as previously documented (Rittenbach and Baker, 2007). The medium consisted of 25 μl of an NADPH-generating system (4 mg/ml β-nicotine adenine dinucleotide phosphate, 4 mg/ml glucose-6-phosphate, 3.2 units/ml of glucose-6-phosphate dehydrogenase and 2.6 mg/ml of magnesium chloride in 0.1M potassium phosphate buffer (pH=7.4)), 10 µl of microsomal solution (cDNA-expressed 2B6, 2C19 or 2D6), 10.0 µl of MPPT solution (1mg/ml or 1 mg/10 ml) or 9.3 µl of (-)-deprenyl solution (1 mg/ml or 1 mg/10 ml) (resulting in final concentrations of 500 µM and 50 µM respectively) and sufficient buffer to result in a volume of 100 µl. The CYP content of the cDNA-expressed microsomes was 88, 26 and 110 pmol/mg for 2B6, 2C19 and 2D6 respectively. (-)-Deprenyl had been previously run with each of the enzymes and was used as the positive control for these experiments (Rittenbach and Baker, 2007). The metabolic reaction was allowed to proceed for 120 min in a 37°C water bath, after which the samples were placed on ice and 50 µl of 25% potassium carbonate solution were added to stop the reaction.

Double-distilled water (850 µl) and 30 µl of working IS solution (300 ng) were added and the samples were transferred to screw top tubes.

7.2.3 Sample preparation, derivatization and analysis

The analysis of samples for metabolites of MPPT and (-)-deprenyl has been described elsewhere (see chapters 5 and 6 of this thesis). Briefly, the aqueous samples are basified and shaken with a solution of PFBC in ethyl acetate for analysis of all but methamphetamine which was shaken with PFBSC in ethyl acetate:acetonitrile. The organic phase is retained and taken to dryness. The residue is taken up in toluene, and an aliquot of this solution is analyzed using a gas chromatograph equipped with a capillary column and an electron-capture detector.

7.2.4 Statistics

All interactions were analyzed by two way ANOVA calculated with GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) followed, where necessary, by Bonferroni post tests.

7.3 RESULTS

The results from incubating MPPT and (-)-deprenyl with CYP2B6 (Figure 7.3), CYP2C19 (Figure 7.4) and CYP2D6 (Figure 7.5) at 50 μM and 500 μM for 120 minutes at 37°C are summarized in Figures. 7.3-7.5.

CYP2B6 catalyzed the depropargylation and demethylation of both MPPE and (-)-deprenyl at 500 μM, but did not catalyze the demethylation of MPPT at 50 μM. CYP2C19 catalyzed the N-depropargylation and N-demethylation of both drugs at both concentrations, although the increase in both pathways was greater for MPPT versus (-)-deprenyl when the concentrations were increased from 50 μM to 500 μM. CYP2D6 catalyzed the depropargylation and demethylation of (-)-deprenyl, but does not catalyze either for MPPT. All of the metabolites were produced in significantly greater amounts in the 500 μM incubation versus the 50 μM incubation except for the metabolites of (-)-deprenyl formed by CYP2C19.

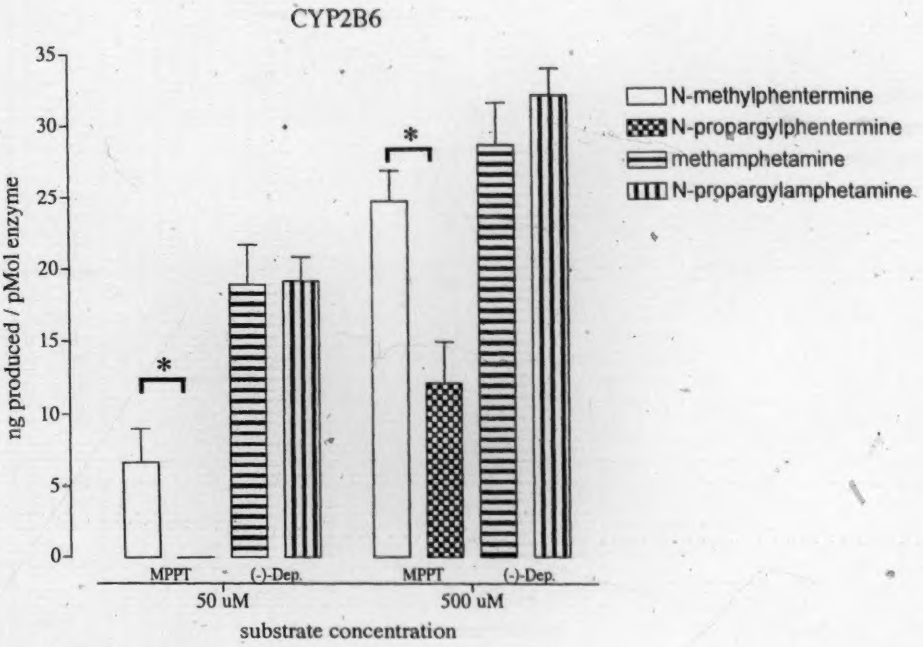


Figure 7.3. Results of MPPT and (-)-deprenyl incubations with CYP2B6 at 50 μM and 500 μM. Results are expressed as means ± SEM (N=4). * = $p < 0.05$.

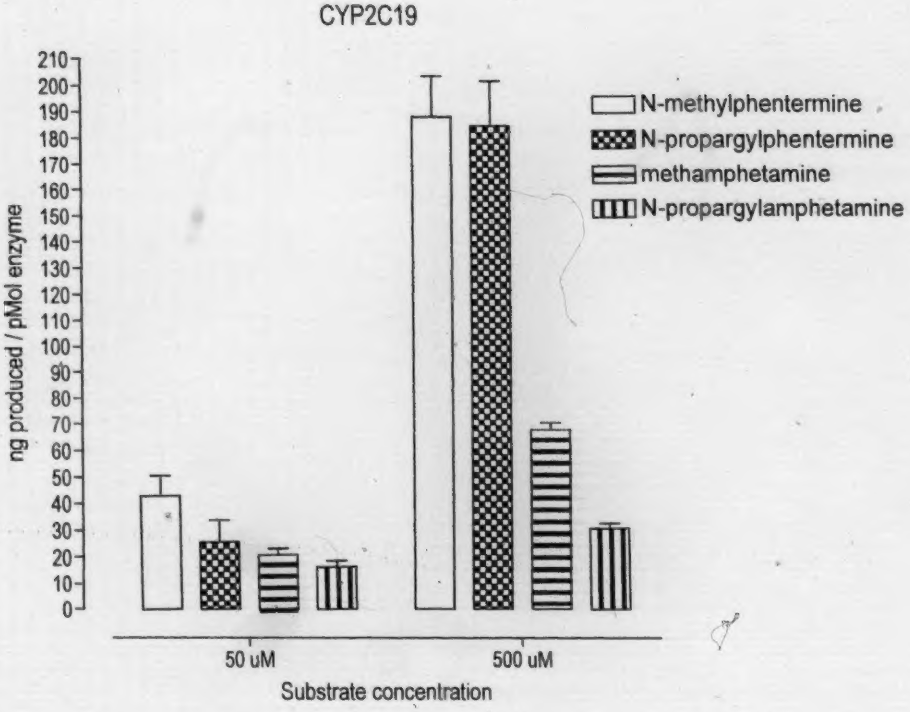


Figure 7.4. Incubation of MPPT and (-)-deprenyl with CYP2C19 results. Results are expressed as means ± SEM (N=4 for MPPT and N=10 for (-)-deprenyl).

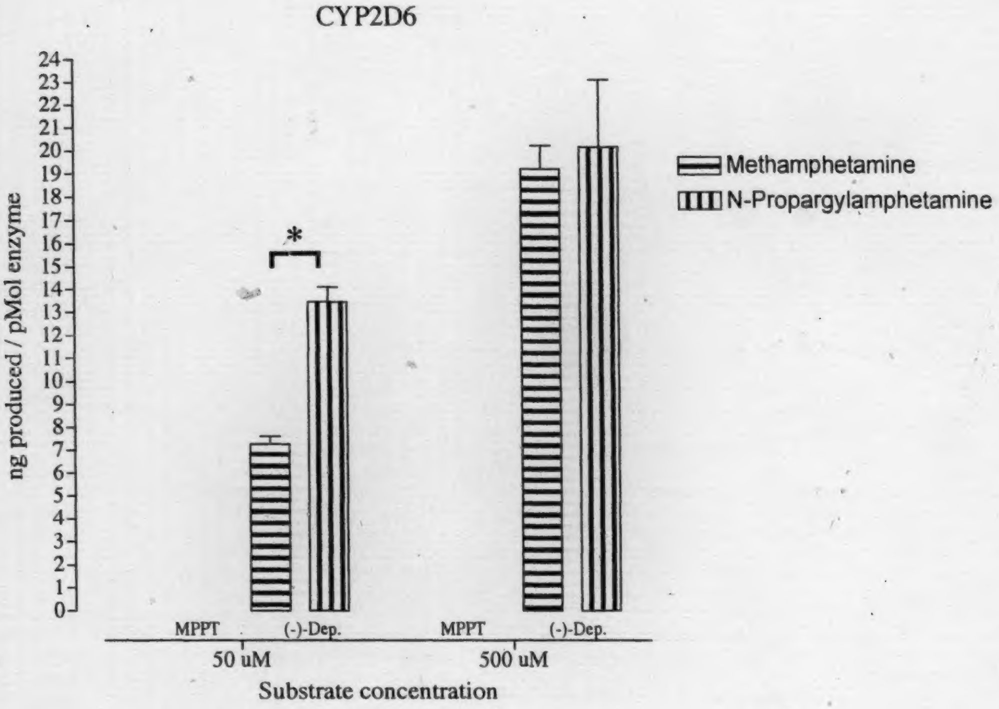


Figure 7.5. Incubation results of MPPT and (-)-deprenyl with CYP2D6. Results are expressed as means ± SEM (N=4 for MPPT and N=8 for (-)-deprenyl). * = $p < 0.05$. CYP2D6 had no effect on metabolism of MPPT.

7.4 DISCUSSION

The differences between the metabolic profiles of these two drugs are of interest in part to help elucidate (-)-deprenyl metabolic pathways but more so because the chemical structures are very similar and the metabolic differences would appear to be the result of a single change at the α carbon, i.e. the addition of a methyl group in MPPT.

According to our findings reported here, formation of N-propargylamphetamine from (-)-deprenyl can be catalyzed by CYP2D6 and CYP2B6. The formation of N-propargyamphetamine catalyzed by CYP2C19 was low and variable and the amount formed did not differ between (-)-deprenyl concentrations of 50 and 500 μM. The reasons for these effects with CYP2C19 are not clear at this time.

One striking difference in the metabolism of these compounds is that CYP2D6 catalyzes both the N-demethylation and N-depropargylation of (-)-deprenyl, but does not catalyze either reaction with MPPT even though it did show interactions in the fluorometric screen. This suggests that MPPT is an inhibitor but not a substrate for CYP2D6.

It is clear from our results that MPPT is metabolized similarly to (-)-deprenyl, i.e. both N-demethylation and N-depropargylation occur. However, the marked differences found with CYP2D6 are interesting in lieu of the fact that the two drugs differ from one another only in the presence of an additional α-methyl group in MPPT.

7.5 REFERENCES

Akhondzadeh, S., Tavakolian, R., Davari-Ashtiani, R., Arabgol, F. and Amini, H. (2003) Selegiline in the treatment of attention deficit hyperactivity disorder in children: a double blind and randomized trial. *Prog Neuro-Psychopharmacol and Biol Psychiatry* 27:841-845

Bach, M. V., Coutts, R. T. and Baker, G. B. (2000) Metabolism of N,N-dialkylated amphetamines, including deprenyl, by CYP2D6 expressed in a human cell line. *Xenobiotica* 30:297-306

Callahan, B. T., Yuan, J. and Ricaurte, G. A. (2000) Fluoxetine increases the anorectic and long-term dopamine-depleting effects of phentermine. *Synapse* 38:471-6

DeLaGarza, V. W. (2003) Pharmacologic treatment of Alzheimer's disease: an update. *Am Fam Physician* 68:1365-72

Elkashef, A., Fudala, P. J., Gorgon, L., Li, S. H., Kahn, R., Chiang, N., Vocci, F., Collins, J., Jones, K., Boardman, K. and Sather, M. (2006) Double-blind, placebo-controlled trial of selegiline transdermal system (STS) for the treatment of cocaine [illegible]ence. *Drug Alcohol Depend* 85:191-7

Glazer, G. (2001) Long-term pharmacotherapy of obesity 2000: a review of efficacy and safety. *Archives of internal medicine* 161:1814-24

Grace, J. M., Kinter, M. T. and Macdonald, T. L. (1994) Atypical metabolism of deprenyl and its enantiomer, (S)-(+)-N,alpha-dimethyl-N-propynylphenethylamine, by cytochrome P450 2D6. *Chem Res Toxicol* 7:286-90

Hidestrand, M., Oscarson, M., Salonen, J. S., Nyman, L., Pelkonen, O., Turpeinen, M. and Ingelman-Sundberg, M. (2001) CYP2B6 and CYP2C19 as the major enzymes responsible for the metabolism of selegiline, a drug used in the treatment of Parkinson's disease, as revealed from experiments with recombinant enzymes. *Drug Metab Dispos* 29:1480-1484

Kim, K. K., Cho, H. J., Kang, H. C., Youn, B. B. and Lee, K. R. (2006) Effects on weight reduction and safety of short-term phentermine administration in Korean obese people. *Yonsei Med J* 47:614-25

Mandel, S., Grunblatt, E., Riederer, P., Gerlach, M., Levites, Y. and Youdim, M. B. (2003) Neuroprotective strategies in Parkinson's disease : an update on progress. *CNS Drugs* 17:729-62

McCann, D. J., Mello, N. K., Negus, S. S., Bergman, J., Forster, M. and Schenk, S. (1999) Selegiline as a potential cocaine treatment medication: behavioral evaluation in rhesus monkeys and comparisons with other MAO inhibitors in rodents. *Behav Pharmacol* 10:S59-S60

McCann, U. D., Yuan, J. and Ricaurte, G. A. (1998) Neurotoxic effects of +/- fenfluramine and phentermine, alone and in combination, on monoamine neurons in the mouse brain. *Synapse* 30:239-46

Olanow, C. W. (2004) The scientific basis for the current treatment of Parkinson's disease. *Ann Rev Med* 55:41-60

Paterson, I. A., Zhang, D., Warrington, R. C. and Boulton, A. A. (1998) R-deprenyl and R-2-heptyl-N-methylpropargylamine prevent apoptosis in cerebellar granule neurons induced by cytosine arabinoside but not low extracellular potassium. *J Neurochem* 70:515-23

Patkar, A. A., Pae, C. U. and Masand, P. S. (2006) Transdermal selegiline: the new generation of monoamine oxidase inhibitors. *CNS Spectr* 11:363-75

Riederer, P. and Lachenmayer, L. (2003) Selegiline's neuroprotective capacity revisited. *J Neural Transm* 110:1273-8

Rittenbach, K. and Baker, G. B. (2007) Involvement of cytochromes P450 2D6, 2B6 and 2C19 in the metabolism of (-)-deprenyl and N-methyl,N-propargylphenylethylamine. *Drug Metab Let* 1:97-100

Schapira, A. H. V. and Olanow, C. W. (2004) Neuroprotection in Parkinson disease: Mysteries, myths, and misconceptions. *J Am Med Assoc* 291:358-364

Scheinin, H., Anttila, M., Dahl, M. L., Karnani, H., Nyman, L., Taavitsainen, P., Pelkonen, O. and Bertilsson, L. (1998) CYP2D6 polymorphism is not crucial for the disposition of selegiline. *Clin Pharmacol Ther* 64:402-11

Semkova, I., Wolz, P., Schilling, M. and Krieglstein, J. (1996) Selegiline enhances NGF synthesis and protects central nervous system neurons from excitotoxic and ischemic damage. *Eur J Pharmacol* 315:19-30

Sharma, U., Roberts, E. S. and Hollenberg, P. F. (1996) Inactivation of cytochrome P4502B1 by the monoamine oxidase inhibitors R-(-)-deprenyl and clorgyline. *Drug Metab Dispos* 24:669-75

Shin, H. S. (1997) Metabolism of selegiline in humans. Identification, excretion, and stereochemistry of urine metabolites. *Drug Metab Dispos* 25:657-62

Sowa, B. N., Todd, K. G., Tanay, V. A. M. I., Holt, A. and Baker, G. B. (2004) Amine oxidase inhibitors and development of neuroprotective drugs. *Curr Neuropharmacol* 2:153-168

Stocchi, F. and Olanow, C. W. (2003) Neuroprotection in Parkinson's disease: clinical trials. *Ann Neurol* 53 Suppl 3:S87-97; discussion S97-9

Taavitsainen, P., Anttila, M., Nyman, L., Karnani, H., Salonen, J. S. and Pelkonen, O. (2000) Selegiline metabolism and cytochrome P450 enzymes: in vitro study in human liver microsomes. *Pharmacol Toxicol* 86:215-21

Tabi, T., Magyar, K. and Szoko, E. (2003) Chiral characterization of deprenyl-N-oxide and other deprenyl metabolites by capillary electrophoresis using a dual cyclodextrin system in rat urine. *Electrophoresis* 24:2665-73

Tatton, W. G. (1993) Selegiline can mediate neuronal rescue rather than neuronal protection. *Movement Disord* 8 Suppl 1:S20-30

Tatton, W. G. and Chalmers-Redman, R. M. (1996) Modulation of gene expression rather than monoamine oxidase inhibition: (-)-deprenyl-related compounds in controlling neurodegeneration. *Neurol* 47:S171-83

Thomas, T. (2000) Monoamine oxidase-B inhibitors in the treatment of Alzheimer's disease. *Neurobiol Aging* 21:343-348

Todd, K. G. and Butterworth, R. F. (1998) Increased neuronal cell survival after L-deprenyl treatment in experimental thiamine deficiency. *J Neurosci Res* 52:240-6

Wacher, V. J., Wong, S., Wong, H. T. and Benet, L. Z. (1996) Contribution of CYP3A to selegeline metabolism in rat and human liver microsomes. *Proc. North Am. Meet. Int. Soc. Study Xenobiot., San Diego*

CHAPTER 8

GENERAL DISCUSSION

8.1 DISCUSSION

The studies that comprise the present thesis were designed to investigate the metabolism in humans of two novel neuroprotective drugs [N-methyl,N-propargylphenylethylamine (MPPE) and N-methyl,N-propargylphentermine (MPPT)] that are structural analogues of (-)-deprenyl. The research project involved development of appropriate assays for the metabolies, studying the metabolites in HLMs, screening for likely CYP enzymes involved in the metabolism using a fluorogenic screen, and then focusing on specific CYP enzymes using cDNA-expressed forms of those enzymes. (-)-Deprenyl is a selective irreversible inhibitor of MAO-B, has neuroprotective/neurorescue properties and is commonly prescribed for treatment of Parkinson's Disease (Stocchi and Olanow, 2003). It is also under investigation as treatment for Alzheimer's Disease (Sano *et al.*, 1997; Thomas, 2000; DeLaGarza, 2003), the negative symptoms of schizophrenia (Bodkin *et al.*, 2005; Murphy *et al.*, 2006), and in cocaine and methamphetamine addiction (Bartzokis *et al.*, 1999; Newton *et al.*, 1999; Kosten *et al.*, 2002). However, a distinct disadvantage of (-)-deprenyl is its metabolism to two potential neurotoxic metabolites, (-)-methamphetamine and (-)-amphetamine. This problem has stimulated interest in developing other neuroprotective N-propargylamines that are not metabolized to amphetamines, e.g. rasagiline (Weinreb *et al*, 2005), aliphatic N-propargylamines (Yu *et al.*, 1992) MPPE and MPPT (unpublished communication).

Although it is an MAO-B inhibitor, current research shows many more complex and diverse mechanisms of action for (-)-deprenyl. Antiapoptotic effects are measurable at concentrations lower than the concentrations required to significantly inhibit MAO-B, and further, can be observed in cell lines that do not express MAO-B (Maruyama and Naoi, 1999; Berry, 2004). The proposed mechanisms of action for (-)-deprenyl have been discussed at length in the introduction of this thesis and so will not be reiterated here. Three of particular interest, however, are the increases in cell/cell adhesion (Jenei *et al.*, 2005), direct action on the mitochondrial membrane potential (Simon *et al.*, 2005) and destabilization of β-amyloid fibrils (Ono *et al.*, 2006). These mechanisms of action have all been reported recently for (-)-deprenyl and all are implicated in neurodegeneration.

The major reason that structural analogues of (-)-deprenyl have been synthesized and tested for neuroprotective properties is that (-)-deprenyl is metabolized to (-)-methamphetamine and (-)-amphetamine. (-)-Methamphetamine has been shown to be neurotoxic at low concentrations (Am *et al.*, 2004; McCann *et al.*, 2004), which would work against the desired action of the drug. Further, both (-)-amphetamine and (-)-methamphetamine have abuse potential, although the (-) enantiomers are less addictive than the corresponding (+) enantiomers (Gerlach *et al.*, 1996). Another major metabolite of (-)-deprenyl, N-propargyl-amphetamine, appears to retain neuroprotective traits (Stocchi and Olanow, 2003), which suggests that the N-propargyl group is necessary for neuroprotective actions of these compounds (Maruyama and Naoi, 1999). This is supported by research into the neuroprotective actions of rasagline (N-propargylaminoindan), an N-propargylamine designed to retain neuroprotective properties of (-)-deprenyl but not have neurotoxic metabolites (Maruyama and Naoi, 1999; Weinreb *et al.*, 2005). In addition a series of long-chain aliphatic propargyl-amines which are MAO-B inhibitors have been shown to be neuroprotective (Yu *et al.*, 1992; Berry and Boulton, 2002), as have the N-propargylamines clorgyline, a selective MAO-A inhibitor (Malorni *et al.*, 1998) and CGP 3466 (does not inhibit MAO) (Youdim *et al.*, 2006). The structural analogues studied in this thesis project were synthesized because they retain the N-propargyl group, the theorized metabolites were not amphetamines, and they provide an interesting structure activity comparison with (-)-deprenyl. Previous work demonstrated that they are neuroprotective (Kwan *et al.*, 2000; unpublished communication) and the work in this thesis has established that, similar to (-)-deprenyl, they undergo N-demethylation and N-depropargylation.

It is now well established in the literature that the N-propargyl group of several N-propargylarylalkylamines is readily removed by metabolism. This phenomenon has been reported with (-)-deprenyl and rasagiline in a variety of studies in rodents and humans (Reynold *et al.*, 1978; Yoshida *et al.*, 1986; Heinonen *et al.*, 1989; Kalasz *et al.*, 1990; Shin, 1997; Bach *et al.*, 2000; Am *et al.*, 2004). N-Depropargylation has also been reported in a number of biological systems with the MAO inhibitor pargyline (N-propargyl,N-methylbenzylamine) (Durden *et al.*, 1975; Pirisino *et al.*, 1978; Coutts *et al.*, 1981; Weli and Lindeke, 1985, 1986). The N-propargyl group has also been added to arylalkylamines such as ß-phenylethylamine and tryptamine to produce prodrugs of these amines in an effort to increase their brain levels (Rao, 1987; Rao *et al.*, 1987a, b).

The first challenge of determining the CYP-mediated metabolism for the novel compounds was establishing sensitive analytical procedures for the six proposed metabolites. Aqueous derivatizations of amines with PBFSC and PFBC are well established techniques for the extractive derivatization and analysis of compounds containing amine and/or phenol groups (Sentissi *et al.*, 1984; Baker *et al.*, 1986a, b; Nazarali *et al.*, 1987a, b; Urichuk *et al.*, 1997; Salsali *et al.*, 2000; Liu *et al.*, 2002; Chou *et al.*, 2004; Xie *et al.*, 2004). This thesis demonstrates the utility of these procedures for the analysis of MPPE and MPPT metabolites.

Comprehensive investigations on the suitability of PFBSC and PFBC as derivatizing agents for the metabolites of MPPE and MPPT were conducted. The metabolites of MPPE were easily derivatized and extracted under aqueous conditions with PFBSC, but the metabolites of MPPT showed better reproducibility with PFBC. N-MethylPHEN and N-propargylPHEN required a higher pH environment for derivatization than did N-methylPEA and N-propargylPEA and even then the sensitivities were lower than for the metabolites of MPPE (see chapter 5 of this thesis). In order to achieve appropriate sensitivities and to analyze all three metabolites at once without interference from other substances present in the extracts, N-methylPHEN, N-propargylPHEN and PHEN had to be derivatized under anhydrous conditions. This may be due to the increased steric hindrance at the active site of derivatization due to two methyl groups on the alpha carbon of the MPPT metabolites, although it is unlikely that this would differ between aqueous and anhydrous conditions. Under the anhydrous conditions, in which the samples are first extracted with organic solvent containing no derivatizing reagent, interfering substances present in the aqueous phase will not have been extracted and derivatized with the MPPT metabolites, which probably accounts for the cleaner samples and improved sensitivity.

The study of enzyme kinetics is a large field that cannot be properly explored in this thesis. The two constants that are most useful in describing metabolism are V_{max} and K_M. V_{max} is the maximum rate of formation of a metabolite when all of the enzyme active sites are full, and K_M is the concentration of substrate that gives exactly one half of the rate of formation of V_{max}. These two constants are used to describe and compare substrates of enzymes. They can be used to compare two or more substrates and determine which will be preferentially metabolized by an enzyme. The substrate with the lowest K_M binds more strongly and will inhibit the metabolism of other substrates.

In this thesis, metabolite formation for the CYP-mediated metabolism of MPPE and MPPT were studied using the analytical procedures developed and individual HLM panels (Rittenbach *et al.*, 2005). The methodology employed was in *in vitro* incubation of the drugs with HLMs in solutions that mimic physiological environments in both pH and ionic strength. The use of HLM panels is a well established *in vitro* technique for metabolism studies with well discussed advantages and disadvantages (Iwatsubo *et al.*, 1997; Venkatakrishnan *et al.*, 2001; Plant, 2004). Some of the advantages are the low cost, simplicity of use, extensive characterization and commercial availability (Brandon *et al.*, 2003). Due to the processing of the HLM samples, CYP enzymes are concentrated and other endogenous enzymes excluded, leading to a lack of competition and therefore potentially increased biotransformation by CYP enzymes in microsomes versus in human *in vivo* situations (Brandon, *et al.*, 2003). At the present time, there is not *in vivo* information available about the levels of the drugs of interest in this thesis in tissue of either laboratory animals or human subjects, so it is not possible to conduct studies *in vitro* at drug concentrations that we are certain are relevant *in vivo*. In fact, there is a paucity of such *in vivo* data even with (-)-deprenyl.

Unfortunately MPPT and MPPE were studied using different individuals (due to the first panel being unavailable from the supplier for the second study) and so the V_{max} and K_M values would not be directly comparable, but the results do provide novel and useful information about the metabolic pathways of these novel compounds.

K_M and V_{max} values are calculated through non-linear regression with the Michaelis-Menten equation. However, this equation is only appropriate when a single enzyme catalyzes the metabolism of the compound. N-PropargylPEA is formed from MPPE by a single CYP enzyme, allowing the calculation of kinetic constants for its formation. N-PropargylPEA formation had a K_M of 290 μM and a V_{max} of 139 ng/ml/min. The values for N-methylPEA formation were not determined experimentally because three enzymes contribute to the formation and hence the equation was too complex for a good fit. The formation of both N-methylPHEN and N-propargylPHEN from MPPT is catalyzed by two enzymes

(CYP2B6 and 2C19) which also complicated the equation significantly and did not allow for the determination of these constants with confidence.

Drug-drug interactions can be potentially life threatening. There are *in vitro* screening methods for drug-drug interactions, based on the principle that if a compound inhibits the metabolism of one enzyme substrate, it will inhibit the metabolism of all substrates of that enzyme. With the recent availability of fluorogenic substrates for most of the human drug-metabolizing CYP enzymes, high throughput fluorescence procedures have become common as a screening method for measuring interactions between compounds and CYP enzymes (Crespi and Stresser, 2000). The procedure demonstrates that the compound being studied (the drug of interest) inhibits the metaboism of the fluorogenic substrate, but it does not determine if the drug of interest is metabolized by that enzyme. It may be a non-competitive inhibitor of the enzyme instead. However, the fluorogenic method is useful as a screening procedure not only because the drug of interest is likely metabolized by an enzyme that it inhibits but also because if the drug is an inhibitor of, but not a substrate for the enzyme, this can still result in adverse drug-drug interactions clinically.

The fluorometric screen used in the projects described in this thesis established that both MPPE and MPPT interacted most strongly with CYP2B6 , 2C19 and 2D6 of the CYP enzymes tested. Although the same CYP enzymes were inhibited, MPPE interacted most strongly with CYP2B6 and MPPT interacted most strongly with CYP2C19.

A study utilizing cDNA-expressed CYP2D6 showed that (-)-deprenyl was an "atypical" substrate of CYP2D6 (Grace *et al.*, 1994). This study demonstrated the formation of both methamphetamine and N-propargylamphetamine by CYP2D6. Further studies supported the involvement of CYP2D6 (Sharma *et al.*, 1996) and also CYP3A4 (Wacher *et al.*, 1996). The study that demonstrated CYP3A4 involvement utilized ketoconazole as a selective CYP3A4 inhibitor, but because this compound also inhibits CYP2B6 and CYP1A2, the involvement of CYP3A4 is still debated (Anttila *et al.*, 2005). Both MPPE and MPPT had only very weak effects on CYP3A4 in the fluorogenic screen, and so this enzyme was not pursued further with regard to their metabolism in my investigation. A study of (-)-deprenyl metabolism in extensive and poor metabolizers of CYP2D6 substrates did not demonstrate a change in metabolism between the two groups, which implies that CYP2D6 is not involved in the clearance of (-)-deprenyl (Scheinin *et al.*, 1998). However, a later study utilizing cDNA-expressed CYP2D6 again indicated its involvement in the metabolism of (-)-deprenyl (Bach *et al.*, 2000). Interestingly, in a study comparing different *in vitro* methods for predicting the CYP enzymes involved in xenobiotic metabolism, the homology modeling method (this method utilizes computer models of the enzyme active sites to predict metabolism of novel compounds) predicted that CYP2D6 would be involved in N-depropargylating (-)-deprenyl, whereas cDNA-expressed enzyme and HLM methods showed no involvement of CYP2D6 (Salonen *et al.*, 2003). These findings with cDNA-expressed CYP2D6 are in contrast to my own and those of Bach *et al.* (2000). The conflicting information about CYP2D6 involvement may be a result of the fact that (-)-deprenyl can be metabolized by CYP2D6, as demonstrated in several of the above mentioned studies and chapters 4 and 7 of this thesis, but that it is preferentially metabolized by other enzymes such as CYP2B6 and CYP2C19. As a result, in human trials and HLM studies, CYP2D6 is not a major route of metabolism and so poor metabolizers do not demonstrate different metabolic profiles than extensive metabolizers and inhibition with selective CYP2D6 inhibitors does not change the metabolic profile significantly.

Although we found that (-)-deprenyl was a substrate for CYP2C19, the results were extremely variable, and the levels of metabolites formed did not differ at the two concentrations of (-)-deprenyl. Both Salonen *et al.* (2003) and Hidestrand *et al.* (2001), in studies using cDNA-expressed enzymes, reported that CYP2C19 was involved in formation of both methamphetamine and N-propargylamphetamine. Neither of these investigators compared activity at two different concentrations of (-)-deprenyl, and both used a concentration (1mM) twice as high as the highest concentration I studied. However, the reasons behind any discrepancies are unclear at this time.

The other enzymes implicated in (-)-deprenyl metabolism include CYPs 2B6, 2C19, 1A2 and 3A4. Both CYP1A2 and 3A4 were identified in studies using HLMs (Taavitsainen *et al.*, 2000). In that study, selective inhibitors of several CYP enzymes were added to the incubation mixtures and changes in the metabolites produced were studied (Taavitsainen *et al.*, 2000). The results showed that CYP1A2 was involved in N-demethylation of (-)-deprenyl, but the inhibitor used for CYP1A2 was fluvoxamine which also inhibits CYPs 2C19 and 3A4. This is of interest because later studies identified CYP2C19 as an important enzyme in the metabolism of (-)-deprenyl, implying that the conclusion that CYP1A2 is involved may be flawed (Hidestrand *et al.*, 2001). The support of involvement of CYP3A4 suffers the same problem since the inhibitor used, ketoconazole, also inhibits CYP2B6 which has been shown to be important in the metabolism of (-)-deprenyl. MPPE and MPPT had only weak inhibitory effects on CYPs 1A2 and 3A4 in the fluorogenic screen and were not investigated further in my studies.

Hidestrand *et al.* (2001) reported that the two enzymes most important in metabolism of (-)-deprenyl are CYPs 2B6 and 2C19; although they also reported metabolism by CYPs 3A4 and 1A2, these two enzymes were much less active towards (-)-deprenyl. Recent work has also reported that CYP2B6 and 2C19 are of importance in (-)-deprenyl metabolism (Salonen *et al.*, 2003). This is of interest clinically in part because CYP2B6 and CYP2C19 are polymorphic which may explain some of the variability in clearance of (-)-deprenyl. CYP2B6 has also been shown to be inducible by phenobarbitals which may result in increased clearance of (-)-deprenyl in some patients (Anttila *et al.*, 2005).

The metabolites of MPPE and MPPT were also included in the fluorogenic screening procedure, although they were not investigated further with the individual CYP enzymes. Interestingly, N-methylPEA only interacted with CYP2D6, while N-propargylPEA interacted with all of the enzymes tested to some degree. In contrast, N-propargylPHEN only interacted with CYP2D6 (and weakly with 1A2) while N-methylPHEN interacted with CYPs 2B6, 2C19 and 2D6.

The current thesis objective was to examine CYP enzymes catalyzed the N-demethylation and N-depropargylation of MPPE and MPPT, and so the cDNA-expressed enzymes chosen for further study were based on the inhibition constants of MPPE and MPPT obtained in the fluorogenic screen. The results of these studies with fluorogenic substrates are summarized qualitatively in Table 8.1. The enzymes that were inhibited most strongly by MPPE and MPPT, i.e. CYPs 2B6, 2C19 and 2D6, were studied individually, incubating them with the same concentrations of MPPE, MPPT and (-)-deprenyl for the same time interval to establish which of the metabolites were formed by which enzyme.

Incubations with cDNA-expressed CYP2B6, 2C19 and 2D6 enzymes established specifically which reactions were catalyzed by these enzymes and showed that small structural changes (i.e. the deletion or addition of an α-methyl group to deprenyl in the case of MPPE and MPPT, respectively) did change the metabolic profile of the compounds quite markedly. CYP2B6 catalyzed the N-demethylation and N-depropargylation all three compounds. CYP2C19 catalyzed the N-demethylation of MPPT and (-)-deprenyl (but not of MPPE) and N-depropargylation of MPPT, MPPE and (-)-deprenyl. CYP2D6 catalyzed the N-demethylation of (-)-deprenyl and the N-depropargylation of (-)-deprenyl and MPPE but not MPPT (summarized in Table 8.2). Although CYP2D6 is generally associated with metabolic mechanisms such as hydroxylation and O-desmethylation, there is also an extensive literature demonstrating

	CYP1A2	CYP2B6	CYP2C9	CYP2C19	CYP2D6	CYP3A4
MPPE	Weak	Strong	Weak	Medium	Medium	Weak
N-methyl-PEA	No interaction	No interaction	No interaction	No interaction	Strong	No interaction
N-propargyl-PEA	Medium	Medium	No interaction	Weak	Strong	Medium
PEA	Medium	Strong	No interaction	No interaction	Medium	Weak
MPPT	Weak	Srong	Weak	Strong	Medium	Weak
N-methyl-PHEN	Medium	Strong	Weak	Medium	Medium	Medium
N-propargyl-PHEN	Medium	Weak	No interaction	Medium	Strong	Medium
phentermine	No interaction	Weak	No interaction	Medium	Strong	Medium

Table 8.1. Summary of fluorogenic screen interactions results based on K_i values determined for each of the parent drugs and metabolites (N=4).

Enzyme	Substrate	Metabolite formed
CYP2B6	MPPE	N-methylphenylethylamine N-propargylphenylethylamine
	MPPT	N-methylphentermine N-propargylphentermine
	(-)-Deprenyl	methamphetamine N-propargylamphetamine
CYP2C19	MPPE	N-methylphenylethylamine
	MPPT	N-methylphentermine N-propargylphentermine
	(-)-Deprenyl	methamphetamine N-propargylamphetamine
CYP2D6	MPPE	N-methylphenylethylamine
	MPPT	-----
	(-)-Deprenyl	methamphetamine N-propargylamphetamine

Table 8.2. Summary of CYP-mediated metabolism of MPPE, MPPT and (-)-deprenyl by CYP2B6, 2C19 and 2D6.

its involvement with N-dealkylation [see references to deprenyl metabolism in this thesis and the review by Coutts *et al.* (1994)]. Interestingly, Bach *et al.* (2000) incubated four N-alkylated methamphetamines [N-allyl,N-methylamphetamine, N-methyl,N-propylamphetamine, N,N-diallylamphetamine and N-benzyl,N-methylamphetamine and (-)-deprenyl] at the same concentration and time with human CYP2D6 and found that the amount of N-demethylation was greatest with (-)-deprenyl. In agreement with my results, Bach *et al.* (2000) also found that more N-propargylamphetamine than methamphetamine was formed from (-)-deprenyl under these conditions.

This novel information about the involvement of various CYP enzymes in the metabolism of these drugs may be useful in the rapidly expanding field of enzyme active site modeling because of the structural similarities among the three compounds. In addition, the findings give valuable clues about the likelihood of drug-drug interactions between these two novel compounds (MPPE and MPPT) and other drugs which could be coadministered in clinical situations.

The present work is the first study to establish that these structural analogues of (-)-deprenyl, i.e. MPPE and MPPT, are metabolized similarly to (-)-deprenyl, i.e. by N-demethylation and N-depropargylation, but that the degrees of interaction with specific CYP enzymes show quite marked differences. These differences may be in part due to stereoselectivity since (-)-deprenyl is a single isomer of a chiral compound whereas both MPPT and MPPE are non-chiral. In addition, methyl groups are electron donators and their absence or presence on the carbon adjacent to the nitrogen group may affect metabolic reactions at the nitrogen site. Steric hindrance may also play a role since in MPPT there are two methyl groups on the α-carbon, in contrast to (-)-deprenyl and MPPE where there are one and no methyl groups present, respectively.

It is of interest to note that MPPE is metabolized to β-phenylethylamine (PEA), a trace amine in the brain that has been described as a neuromodulator and endogenous enhancer substance (Knoll *et al.*, 1996; Shimazu and Miklya, 2004). Levels of PEA are normally quite low in the brain, however when a MAO-B inhibitor is administered they can rise significantly, as has been shown with (-)-deprenyl (Elsworth *et al.*, 1978). This has been proposed as an important factor in (-)-deprenyl's efficacy in the treatment of PD (Reynolds *et al.*, 1978; Gerlach *et al.*, 1992; Gerlach, *et al.*, 1996). The importance of MPPE having two mechanisms of increasing PEA, i.e. through inhibition of MAO-B and through metabolism, is currently unknown, but it could be of benefit in some disorders as PEA enhances dopamine neurotransmission endogenously (Paterson *et al.*, 1998).

MPPT is ultimately metabolized to PHEN which is a noradrenergic stimulant that has been marketed as an anorexiant in the United States since 1959 (Glazer, 2001; Kim *et al.*, 2006). Some studies have reported damage to dopaminergic neurons in animals from PHEN administration (McCann, *et al.*, 1998; Callahan *et al.*, 2000). However, the damage was only apparent at the highest doses studied and was species-dependent, making translation to humans more difficult.

The differences between the metabolism of the secondary metabolites of MPPE and MPPT to the primary metabolites is interesting. Incubations of N-methylPEA with HLMs did not result in measurable amounts of PEA while N-propargylPEA did. This could be for several reasons. Perhaps N-methylPEA is metabolized to PEA and the PEA produced is rapidly metabolized further by the HLMs, although if this was the case it would be expected to also affect PEA produced from the metabolism of N-propargylPEA. Another explanation may be that N-methylPEA is preferentially hydroxylated. Of the CYP enzymes, N-methylPEA only interacted with CYP2D6 in the fluorogenic screening procedure, and CYP2D6 is known to catalyze the hydroxylation of many compounds. The primary metabolites of MPPT, N-methylPHEN and N-propargylPHEN, both resulted in measurable amounts of PHEN when incubated with HLMs. Although N-methylPEA and N-methylPHEN only differ by two methyl groups, the interactions measured in the fluorogenic screen were very different. N-MethylPEA only interacted with CYP2D6 while N-methylPHEN interacted with all of the tested enzymes and interacted most strongly with CYP2B6.

8.2 **Future directions**

If MPPE and MPPT are developed as neuroprotective agents, future work should include further studies on metabolism (including *in vivo* studies in laboratory animals) and also mechanisms of action. The parent drugs and their major metabolites should be analyzed for hydroxylated metabolites as these have been identified for (-)-deprenyl (Philips, 1981; Coutts *et al.*, 1984; Shin, 1997) and several N-alkylamphetamines in a variety of species *in vivo* and *in vitro* using CYP enzymes (Coutts and Dawson, 1977; Coutts *et al.*, 1978; Bach *et al.*, 2000).

The results of such studies would help establish a more complete picture of the metabolism and pharmacology of these compounds, as several of the hydroxylated metabolites may be physiologically active compounds. In addition, their formation would probably be affected dramatically by coadministration of MPPE or MPPT with other drugs that are inhibitors of CYP2D6, a CYP enzyme which often plays a major role in hydroxylation of phenylalkylamines.

Since N-oxidation of (-)-deprenyl has been reported to occur to some extent, the formation of N-oxide metabolites of MPPE and MPPT should also be studied *in vitro* and *in vivo* in future; authentic standards of the N-oxide of MPPE and MPPT are not currently available to us.

Although CYP1A2 and CYP3A4 were not included in the studies described in this thesis because they did not show up as likely candidates in the fluorogenic screen, they should be studied in future given the reports (although somewhat controversial) of other investigators on (-)-deprenyl metabolism using enzyme inhibitiors. As mentioned previously, the fluorogenic screen does not differentiate inhibitory activity from substrate activity and it is possible (although unlikely) that MPPE and MPPT may be substrates of CYP1A2 and CYP3A4 without showing up as inhibitors in the fluorogenic screen.

It will also be of considerable interest to test the N-demethylated metabolites (N-propargylPEA and N-propargylPHEN) for neuroprotective efficacy since the N-demethylated metabolite of (-)-deprenyl, N-propargylamphetamine, has been reported to have neuroprotective capabilities (Mytilineou *et al.*, 1997; Mytilineou *et al.*, 1998; Maruyama and Naoi, 1999).

Such studies should include an investigation of the anti-apoptotic effects of the drugs. The mechanism of action of the anti-apoptotic action of neuroprotective drugs currently available is debated, and so future studies should include several possibilities. Other propargylamines are known to increase the expression of the anti-apoptotic proteins BCL2, BCL-XL and BCL-W and decrease the expression of the pro-apoptotic proteins BAD and BAX [for review see (Youdim and Weinstock, 2001)], and it would be interesting to know if these novel compounds also regulate these proteins. Studies have also shown an action of (-)-deprenyl on the mitochondrial membrane potential, preventing apoptosis, and this too would be an interesting avenue of investigation with these novel compounds (Simon, *et al.*, 2005). The role of the propargylamines in regulating anti-apoptotic and pro-apoptotic proteins may be mediated by binding to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Tatton *et al.*, 2003) and it would be of interest to see if MPPE, MPPT and their N-propargyl metabolites also bind to this protein and affect its nuclear transport.

There are many interesting avenues of future investigation with these two novel neuroprotective agents that may help elucidate the role N-propargylamines play in neuroprotection, and the results with HLMs and individual cDNA-expressed human CYP enzymes presented in this thesis provide a strong foundation for understanding their metabolism and hopefully for predicting pharmacokinetic drug-drug interactions should these drugs be used as pharmacological tools or clinically as neuroprotective agents in the future.

8.3 REFERENCES

Anttila, M., Sotaniemi, E.A., Pelkonen, O. and Rautio, A. (2005) Marked effect of liver and kidney function on the pharmacokinetics of selegiline. *Clin Pharmacol Ther* 77:54-62

Am, O. B., Amit, T. and Youdim, M. B. H. (2004) Contrasting neuroprotective and neurotoxic actions of respective metabolites of anti-Parkinson drugs rasagiline and selegiline. *Neurosci Lett* 355:169-172

Bach, M. V., Coutts, R. T. and Baker, G. B. (2000) Metabolism of N,N-dialkylated amphetamines, including deprenyl, by CYP2D6 expressed in a human cell line. *Xenobiotica* 30:297-306

Baker, G. B., Koilpillai, M., Nazarali, A. J., Rao, T. S. and Coutts, R. T. (1986a) Gas chromatography of antidepressants and their metabolites as pentafluorobenzoyl and pentafluorobenzenesulfonyl derivatives. *Proc West Pharmacol Soc* 29:291-294

Baker, G. B., Rao, T. S. and Coutts, R. T. (1986b) Electron-capture gas chromatographic analysis of beta-phenylethylamine in tissues and body fluids using pentafluorobenzenesulfonyl chloride for derivatization. *J Chromatogr-Biomed Appl* 381:211-7

Bartzokis, G., Beckson, M., Newton, T., Mandelkern, M., Mintz, J., Foster, J. A., Ling, W. and Bridge, T. P. (1999) Selegiline effects on cocaine-induced changes in medial temporal lobe metabolism and subjective ratings of euphoria. *Neuropsychopharmacol* 20:582-90

Berry, M. D. (2004) Glyceraldehyde-3-phosphate dehydrogenase as a target for small-molecule disease-modifying therapies in human neurodegenerative disorders. *J Psychiatr Neurosci* 29:337-45

Berry, M. D. and Boulton, A. A. (2002) Aliphatic propargylamines as symptomatic and neuroprotective treatments for neurodegenerative diseases. *Neurotoxicol Teratol* 24:667-73

Bodkin, J. A., Siris, S. G., Bermanzohn, P. C., Hennen, J. and Cole, J. O. (2005) Double-blind, placebo-controlled, multicenter trial of selegiline augmentation of antipsychotic medication to treat negative symptoms in outpatients with schizophrenia. *Am J Psychiatry* 162:388-390

Brandon, E. F., Raap, C. D., Meijerman, I., Beijnen, J. H. and Schellens, J. H. (2003) An update on in vitro test methods in human hepatic drug biotransformation research: pros and cons. *Toxicol Appl Pharmacol* 189:233-46

Callahan, B. T., Yuan, J. and Ricaurte, G. A. (2000) Fluoxetine increases the anorectic and long-term dopamine-depleting effects of phentermine. *Synapse* 38:471-6

Chou, S.-L., Yang, M.-H., Ling, Y.-C. and Giang, Y.-S. (2004) Gas chromatography-isotope dilution mass spectrometry preceded by liquid-liquid extraction and chemical derivatization for the determination of ketamine and norketamine in urine. *J Chromatogr B* 799:37-50

Coutts, R.T. and Dawson, G.W. (1977) Urinary excretion of phenolic metabolites of N-(n-propyl)amphetamine in man. *Res Comm Chem Path Pharmacol* 17:349-52

Coutts, R.T., Foster, B.C. and Pasutto, F.M. (1981) Fungal metabolism of (-)-deprenyl and pargyline. *Life Sci* 29:1951-8

Coutts, R.T., Prelusky, D.B. and Baker, G.B. (1984) Determination of amphetamine, norephedrine, and their phenolic metabolites in rat brain by gas chromatography. *J Pharm Sci.* 73:808-12

Coutts, R. T., Su, P. and Baker, G. B. (1994) Involvement of CYP2D6, CYP3A4, and other cytochrome P-450 isozymes in N-dealkylation reactions. *J Pharmacol Toxicol Methods* 31:177-86

Crespi, C. L. and Stresser, D. M. (2000) Fluorometric screening for metabolism-based drug-drug interactions. *J Pharmacol Toxicol Methods* 44:325-31

DeLaGarza, V. W. (2003) Pharmacologic treatment of Alzheimer's disease: an update. *Am Fam Physician* 68:1365-72

Durden, D.A., Philips, S.R. and Boulton A.A. (1975) Identification and distribution of benzylamine in tissue extracts isolated from rats pretreated with pargyline. *Biochem Pharmacol* 24:1-2

Elsworth, J. D., Glover, V., Reynolds, G. P., Sandler, M., Lees, A. J., Phuapradit, P., Shaw, K. M., Stern, G. M. and Kumar, P. (1978) Deprenyl administration in man: a selective monoamine oxidase B inhibitor without the 'cheese effect'. *Psychopharmacology (Berl)* 57:33-8

Gerlach, M., Riederer, P. and Youdim, M. B. (1992) The molecular pharmacology of L-deprenyl. *Eur J Pharmacol* 226:97-108

Gerlach, M., Youdim, M. B. and Riederer, P. (1996) Pharmacology of selegiline. *Neurol* 47:S137-45

Glazer, G. (2001) Long-term pharmacotherapy of obesity 2000: a review of efficacy and safety. *Arch Int Med* 161:1814-24

Grace, J. M., Kinter, M. T. and Macdonald, T. L. (1994) Atypical metabolism of deprenyl and its enantiomer, (S)-(+)-N,alpha-dimethyl-N-propynyl-phenethylamine, by cytochrome P450 2D6. *Chem Res Toxicol* 7:286-90

Heinonen, E.H., Myllyl, V. and Sotaniemi, K. (1989) Pharmacokinetics and metabolism of selegiline. *Acta Neurol Scand* 80(Suppl 126):93-9

Hidestrand, M., Oscarson, M., Salonen, J. S., Nyman, L., Pelkonen, O., Turpeinen, M. and Ingelman-Sundberg, M. (2001) CYP2B6 and CYP2C19 as the major enzymes responsible for the metabolism of selegiline, a drug used in the treatment of Parkinson's disease, as revealed from experiments with recombinant enzymes. *Drug Metab Dispos* 29:1480-1484

Iwatsubo, T., Hirota, N., Ooie, T., Suzuki, H., Shimada, N., Chiba, K., Ishizaki, T., Green, C. E., Tyson, C. A. and Sugiyama, Y. (1997) Prediction of in vivo drug metabolism in the human liver from in vitro metabolism data. *Pharmacol Ther* 73:147-71

Jenei, V., Zor, K., Magyar, K. and Jakus, J. (2005) Increased cell-cell adhesion, a novel effect of R-(-)-deprenyl. *J Neural Transm* 112:1433-45

Kalasz, H., Kerecsen, L., Knoll, J. and Pucsok, J. (1990) Chromatographic studies on the binding, action and metabolism of (-)-deprenyl. *J Chromatogr* 499:589-99

Kim, K. K., Cho, H. J., Kang, H. C., Youn, B. B. and Lee, K. R. (2006) Effects on weight reduction and safety of short-term phentermine administration in Korean obese people. *Yonsei Med J* 47:614-25

Knoll, J., Miklya, I., Knoll, B., Marko, R. and Racz, D. (1996) Phenylethylamine and tyramine are mixed-acting sympathomimetic amines in the brain. *Life Sci* 58:2101-14

Kosten, T. R., George, T. P. and Kosten, T. A. (2002) The potential of dopamine agonists in drug addiction. *Expert Opinion on Investigational Drugs* 11:491-9

Kwan, E., Baker, G. B., Shuaib, A., Ling, L. and Todd, K. G. (2000) N-Methyl,N-propargyl-2-phenylethylamine (MPPE), an analog of deprenyl, increases neuronal cell survival in thiamin deficiency encephalopathy. *Drug Devel Res* 51:244-252

Liu, Z. Q., Tan, Z. R., Wang, D., Huang, S. L., Wang, L. S. and Zhou, H. H. (2002) Simultaneous determination of fluoxetine and its metabolite p-trifluoromethylphenol in human liver microsomes using a gas chromatographic-electron-capture detection procedure. *J Chromatogr B* 769:305-11

Malorni, W., Giammarioli, A. M., Matarrese, P., Pietrangeli, P., Agostinelli, E., Ciaccio, A., Grassilli, E. and Mondovi, B. (1998) Protection against apoptosis by monoamine oxidase A inhibitors. *FEBS letters* 426:155-9

Maruyama, W. and Naoi, M. (1999) Neuroprotection by (-)-deprenyl and related compounds. *Mech Ageing Dev* 111:189-200

McCann, U. D. and Ricaurte, G. A. (2004) Amphetamine neurotoxicity: accomplishments and remaining challenges. *Neurosci Biobehav Rev* 27:821-826

McCann, U. D., Yuan, J. and Ricaurte, G. A. (1998) Neurotoxic effects of (+/-) fenfluramine and phentermine, alone and in combination, on monoamine neurons in the mouse brain. *Synapse* 30:239-46

Murphy, B. P., Chung, Y. C., Park, T. W. and McGorry, P. D. (2006) Pharmacological treatment of primary negative symptoms in schizophrenia: a systematic review. *Schizophrenia Res* 88:5-25

Mytilineou, C., Leonardi, E. K., Radcliffe, P., Heinonen, E. H., Han, S. K., Werner, P., Cohen, G. and Olanow, C. W. (1998) Deprenyl and desmethylselegiline protect mesencephalic neurons from toxicity induced by glutathione depletion. *J Pharmacol Exp Ther* 284:700-6

Mytilineou, C., Radcliffe, P. M. and Olanow, C. W. (1997) L-(-)-desmethyl-selegiline, a metabolite of selegiline [L-(-)-deprenyl], protects mesencephalic dopamine neurons from excitotoxicity in vitro. *J Neurochem* 68:434-6

Nazarali, A.J., Baker, G.B., Coutts, R.T. and Greenshaw, A.J. (1987a) *Para*-hydroxytranylcypormine: presence in rat brain and heart following administration of tranylcypromine and an N-cyanoethyl analogue. *Eur J Drug Metab Pharmacokinet* 12:207-14

Nazarali, A.J., Baker, G.B., Coutts, R.T., Yeung, J.M. and Rao, T.S. (1987b) Rapid analysis of ß-phenylethylamine in tissue and body fluids utilizing pentafluorobenzoylation followed by electron-capture detection. *Progr Neuro-Psychopharmacol Biol Psychiatry* 11:251-8

Newton, T. F., Kalechstein, A., Beckson, M., Bartzokis, G., Bridge, T. P. and Ling, W. (1999) Effects of selegiline pretreatment on response to experimental cocaine administration. *Psychiatry Res* 87:101-6

Ono, K., Hasegawa, K., Naiki, H. and Yamada, M. (2006) Anti-Parkinsonian agents have anti-amyloidogenic activity for Alzheimer's beta-amyloid fibrils in vitro. *Neurochem Int* 48:275-85

Paterson, I. A., Zhang, D., Warrington, R. C. and Boulton, A. A. (1998) R-deprenyl and R-2-heptyl-N-methylpropargylamine prevent apoptosis in cerebellar granule neurons induced by cytosine arabinoside but not low extracellular potassium. *J Neurochem* 70:515-23

Philips, S.R. (1981) Amphetamine, p-hydroxyamphetamine and ß-phenethylamine in mouse brain and urine after (-)- and (+)-deprenyl administration. *J Pharm Pharmacol* 33:739-41

Pirisino, R., Ciottoli, G.B., Buffoni, F., Anselmi, B. and Curradi, C. (1978) N-methylbenzylamine, a metabolite of pargyline in man. *Br J Clin Pharmacol* 7:595-8

Plant, N. (2004) Strategies for using in vitro screens in drug metabolism. *Drug Discovery Today* 9:328-36

Rao, T.S. (1987) Analogs of bioactive arylalkylamines. Neurochemical and neuorpharmacological studies in the rat. PhD thesis, University of Alberta, Edmonton, Canada

Rao, T.S., Baker, G.B. and Coutts, R.T. (1987a) Pharmacokinetic and neurochemical studies on N-propargyl-2-phenylethylamine, a prodrug of 2-phenylethylamine. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 336:25-32

Rao, T.S., Baker, G.B. and Coutts, R.T. (1987b) N,N-Dipropargyl-2-phenylethylamine, a potential prodrug of 2-phenylethylamine: neurochemical and neuropharmacological studies in the rat. *Brain Res Bull* 19:47-55

Reynolds, G. P., Riederer, P., Sandler, M., Jellinger, K. and Seemann, D. (1978) Amphetamine and 2-phenylethylamine in post-mortem Parkinsonian brain after (-) deprenyl administration. *J Neural Transm* 43:271-7

Rittenbach, K., Sloley, B. D., Ling, L., Coutts, R. T., Shan, J. and Baker, G. B. (2005) A rapid, sensitive electron-capture gas chromatographic procedure for analysis of metabolites of N-methyl,N-propargylphenylethylamine, a potential neuroprotective agent. *J Pharmacol Toxicol Methods* 52:373-8

Salonen, J. S., Nyman, L., Boobis, A. R., Edwards, R. J., Watts, P., Lake, B. G., Price, R. J., Renwick, A. B., Gomez-Lechon, M.-J., Castell, J. V., Ingelman-Sundberg, M., Hidestrand, M., Guillouzo, A., Corcos, L., Goldfarb, P. S., Lewis, D. F. V., Taavitsainen, P. and Pelkonen, O. (2003) Comparative studies on the cytochrome P450-associated metabolism and interaction potential of selegiline between human liver-derived in vitro systems. *Drug Metab Dispos* 31:1093-1102

Salsali, M., Coutts, R. T. and Baker, G. B. (2000) A rapid electron-capture gas chromatographic procedure for the analysis of p-hydroxymephenytoin. *J Pharmacol Toxicol Methods* 44:461-5

Sano, M., Ernesto, C., Thomas, R. G., Klauber, M. R., Schafer, K., Grundman, M., Woodbury, P., Growdon, J., Cotman, C. W., Pfeiffer, E., Schneider, L. S. and Thal, L. J. (1997) A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimer's disease. The Alzheimer's Disease Cooperative Study. *N Engl J Med* 336:1216-22

Scheinin, H., Anttila, M., Dahl, M. L., Karnani, H., Nyman, L., Taavitsainen, P., Pelkonen, O. and Bertilsson, L. (1998) CYP2D6 polymorphism is not crucial for the disposition of selegiline. *Clin Pharmacol Ther* 64:402-11

Sentissi, A., Joppich, M., O'Connell, K., Nazareth, A. and Giese, R. W. (1984) Pentafluorobenzenesulfonyl chloride: a new electrophoric derivatizing reagent with application to tyrosyl peptide determination by gas chromatography with electron capture detection. *Anal Chem* 56:2512-7

Sharma, U., Roberts, E. S. and Hollenberg, P. F. (1996) Inactivation of cytochrome P4502B1 by the monoamine oxidase inhibitors R-(-)-deprenyl and clorgyline. *Drug Metab Dispos* 24:669-75

Shimazu, S. and Miklya, I. (2004) Pharmacological studies with endogenous enhancer substances: [beta]-phenylethylamine, tryptamine, and their synthetic derivatives. *Prog Neuro-Psychopharmacol Biol Psychiatry* 28:421-427

Shin, H. S. (1997) Metabolism of selegiline in humans. Identification, excretion, and stereochemistry of urine metabolites. *Drug Metab Dispos* 25:657-62

Simon, L., Szilagyi, G., Bori, Z., Telek, G., Magyar, K. and Nagy, Z. (2005) Low dose (-) deprenyl is cytoprotective: it maintains mitochondrial membrane potential and eliminates oxygen radicals. *Life Sci* 78:225-31

Stocchi, F. and Olanow, C. W. (2003) Neuroprotection in Parkinson's disease: clinical trials. *Ann Neurol* 53 Suppl 3:S87-97; discussion S97-9

Taavitsainen, P., Anttila, M., Nyman, L., Karnani, H., Salonen, J. S. and Pelkonen, O. (2000) Selegiline metabolism and cytochrome P450 enzymes: in vitro study in human liver microsomes. *Pharmacol Toxicol* 86:215-21

Tatton, W., Chalmers-Redman, R. and Tatton, N. (2003) Neuroprotection by deprenyl and other propargylamines: glyceraldehyde-3-phosphate dehydrogenase rather than monoamine oxidase B. *J Neural Transm* 110:509-15

Thomas, T. (2000) Monoamine oxidase-B inhibitors in the treatment of Alzheimer's disease. *Neurobiol Aging* 21:343-348

Urichuk, L. J., Aspeslet, L. J., Holt, A., Silverstone, P. H., Coutts, R. T. and Baker, G. B. (1997) Determination of p-trifluoromethylphenol, a metabolite of fluoxetine, in tissues and body fluids using an electron-capture gas chromatographic procedure. *J Chromatogr B* 698:103-9

Venkatakrishnan, K., Von Moltke, L. L. and Greenblatt, D. J. (2001) Human drug metabolism and the cytochromes P450: application and relevance of in vitro models. *J Clin Pharmacol* 41:1149-79

Wacher, V. J., Wong, S., Wong, H. T. and Benet, L. Z. (1996) Contribution of CYP3A to selegeline metabolism in rat and human liver microsomes. *Proc. North Am. Meet. Int. Soc. Study Xenobiot., San Diego*

Weinreb, O., Amit, T., Bar-Am, O., Chillag-Talmor, O. and Youdim, M. B. (2005) Novel neuroprotective mechanism of action of rasagiline is associated with its propargyl moiety: interaction of Bcl-2 family members with PKC pathway. *Ann N Y Acad Sci* 1053:348-55

Weli, A.M. and Lindeke, B. (1985) The metabolic fate of pargyline in rat liver microsomes. *Biochem Pharmacol* 34:1993-8

Weli, A.M. and Lindeke, B. (1986) Peroxidative N-oxidation and N-dealkylation reactions of pargyline. *Xenobiotica* 16:281-8

Xie, S., Aspromonte, J., Balla, A., Sershen, H., Javitt, D. C. and Cooper, T. B. (2004) Sensitive and simple gas chromatographic-mass spectrometric determination for amphetamine in microdialysate and ultrafiltrate samples. *J Chromatogr B* 805:27-31

Yoshida, T., Yamada, Y., Yamamoto, T. and Kuroiwa, Y. (1986) Metabolism of deprenyl, a selective monoamine oxidase (MAO) B inhibitor in rat:

relationship of metabolism to MAO-B inhibitor potency. *Xenobiotica* 16:129-36

Youdim, M. B., Edmondson, D. and Tipton, K. F. (2006) The therapeutic potential of monoamine oxidase inhibitors. *Nat Rev* 7:295-309

Youdim, M. B. and Weinstock, M. (2001) Molecular basis of neuroprotective activities of rasagiline and the anti-Alzheimer drug TV3326 [(N-propargyl-(3R)aminoindan-5-YL)-ethyl methyl carbamate]. *Cell Mol Neurobiol* 21:555-73

Yu, P. H., Davis, B. A. and Boulton, A. A. (1992) Aliphatic propargylamines: potent, selective, irreversible monoamine oxidase B inhibitors. *J Med Chem* 35:3705-13